Remarks

Reconsideration of this Application is respectfully requested.

Applicants submit herewith a substitute specification, excluding the claims, and a marked-up version of the substitute specification in compliance with 37 C.F.R. §§ 1.52 and 1.125. This substitute specification corrects a minor error in the application regarding the proper name of the receptor and contains no new matter. The amendments to the specification made in the Preliminary Amendment filed March 11, 2004 are also incorporated into the substitute specification and marked-up version.

Upon entry of the foregoing amendment, claims 20 and 22-28 are pending in the application, with claims 20 and 24-28 being the independent claims. Claims 24 and 27 have been amended to clarify that the polynucleotides are variants of SEQ ID NO:1. Claims 20 and 24-28 have been amended to clarify the classification of test compounds and correct an error in the name of the receptor. Support for the amendments can be found throughout the specification, *inter alia*, at page 14, line 21 through page 15, line 9; and page 27, line 1 through page 28, line 9. Support for the proper name of the receptor can be found in Figure 5B. Based on the above amendment and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

Rejections under 35 U.S.C. § 112, first paragraph

Claims 24 and 27 were rejected under 35 U.S.C. 112, first paragraph as allegedly not enabled. Not in acquiescence in the propriety of the rejection, but rather solely to

advance prosecution, Applicants have amended the claims to clarify that the polynucleotides are variants of SEQ ID NO:1. Therefore, Applicants respectfully request that the rejection be reconsidered and removed.

Claims 24 and 27 were rejected under 35 U.S.C. § 112, first paragraph as allegedly failing to comply with the written description requirement. Not in acquiescence in the propriety of the rejection, but rather solely to advance prosecution, Applicants have amended the claims to clarify that the polynucleotides are variants of SEQ ID NO:1. Therefore, Applicants respectfully request that the rejection be reconsidered and removed.

Rejections under 35 U.S.C. § 112, second paragraph

Claims 20 and 22-28 were rejected under 35 U.S.C. § 112, second paragraph as allegedly indefinite for failing to particularly point out and distinctly claim the subject matter regarded as the invention. Not in acquiescence to the propriety of the rejection, but rather solely to advance prosecution, Applicants have amended the claims to clarify that agonist compounds increase cAMP accumulation, while antagonist compounds prevent cAMP accumulation. These amendments are fully supported by the specification. Applicants have also amended the claims to clarify that the proper name of the receptor is rΔNt and not rδNt. The greek Δ signifies the deletion of the extracellular domain. In addition, rΔNt is not an acronym, but rather the scientific name of the receptor. Accordingly, Applicants respectfully request that the rejection be reconsidered and withdrawn.

Rejections under 35 U.S.C. § 102

Claims 20, 22, 23 and 28 were rejected under 35 U.S.C. § 102(b) as allegedly anticipated by U.S. Patent No. 5,494,806 ("the '806 patent"). Applicants respectfully traverse the rejection.

To anticipate a claimed invention, each and every claim limitation must be taught by the prior art. The Examiner alleges that the '806 patent teaches SEQ ID NO:3 which is a nucleic acid sequence encoding an amino acid sequence that is 96.1% identical to SEQ ID NO:2 and provides an alignment of these sequences. However, it must be noted that the Examiner *does not* take into account the deletion of the extracellular amino-terminal ligand binding domain in the alignment, for if this were the case, the percent identity would be vastly reduced because of the absence of 155 amino acids between the two sequences. Since the '806 patent does not disclose an amino acid sequence that is at least ***95% identical to SEQ ID NO:2***, the '806 patent does not teach each and every element of the claimed invention. Accordingly, Applicants respectfully request that the rejection be reconsidered and withdrawn.

Other matters

The Examiner alleges that the information disclosure statement filed May 9, 2003 fails to comply with 37 C.F.R. § 1.98(a)(2) because copies of references AS (Barbier *et al.*) and AT (Chorev *et al.*) were not provided and requests that a new information disclosure statement be submitted. Applicants respectfully assert that copies of the documents were provided and submit a copy of the stamped postcard (Exhibit 1) as proof

of submission of the documents. Therefore, Applicants should not be required to submit a new information disclosure statement *with a fee* to provide documents that were previously provided. For the Examiner's convenience, however, Applicants provide a copy of reference AS (Exhibit 2) and reference AT (Exhibit 3) herewith, and respectfully request that the references be considered and made of record.

Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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Appendix

Attachments: Substitute specification and marked-up version

MARKED-UP VERSION

PTH RECEPTOR AND SCREENING ASSAY UTILIZING THE SAME

Background of the Invention

Statement as to Rights to Inventions Made Under Federally-Sponsored Research and Development

[0001] Part of the work performed during development of this invention utilized U.S. Government funds. The U.S. Government has certain rights in this invention.

Field of the Invention

[0002] The present invention is related to the fields of molecular biology, developmental biology, physiology, neurobiology, endocrinology and medicine.

Related Art

[0003] PTH is the principal regulator of blood calcium levels and mediates this action through binding to PTH-1 receptors on bone and kidney cells (Kronenberg, H.M., *et al.*, in "Handbook of Experimental Pharmacology, Springer-Verlag," Heidelberg (1993)). This receptor also responds to PTH-related peptide, a factor which plays a role in embryonic bone development and is the causative agent of hypercalcemia of malignancy (Lanske, B., *et al., Science 273*:663-666 (1996)). PTH and PTHrP peptides have been shown to have potent anabolic effects on bone, and it is possible, therefore, that PTH-1 receptor agonists could ultimately be used to treat metabolic bone diseases, such as osteoporosis (Dempster, D.W., *et al., Endocr Rev. 14(6)*:690-709 (1994)).

[0004] In the fully bioactive PTH(1-34) peptide, the major determinants of receptor-binding affinity reside within amino acids 15 to 34 (Nussbaum, S.R., *et al., J. Biol. Chem. 255*:10183-10187 (1980); Gardella, T.J., *et al., Endocrinology 132(5)*:2024-2030 (1993); Caulfield, M.P., *et al.,*

Endocrinology 127:83-87 (1990); Abou-Samra, A. B., *et al., Endocrinology 125*:2215-2217 (1989)), which are moderately conserved among PTHs and PTHrPs from various species (Suva, L.J., *et al., Science 237(4817)*:893-896 (1987)). The determinants of receptor activation lie within the more stringently conserved amino-terminal residues, and deletion of these residues yields competitive PTH-1 receptor antagonists (Horiuchi, N., *et al., Science 220*:1053-1055 (1983); Nutt, R.F., *et al., Endocrinology 127*:491-493 (1990)). Amino-terminal PTH or PTHrP fragments shorter in length than PTH(1-27) have not previously been found to be biologically active (Rosenblatt, M., *Pathobilogy Annual*, Raven Press, New York, 11:53-84 (1981); Azarani, A., *et al., J. Biol. Chem. 271(25)*:14931-14936 (1996); Tregear, G.W., *et al., Endocrinology 93*:1349-1353 (1973)), yet the functional importance and evolutionary conservation of the amino-terminal residues predicts that they directly interact with the receptor.

[0005] The PTH-1 receptor couples strongly to the adenylyl cyclase/protein kinase A signaling pathway and, in some settings, to other pathways including those mediated by phospholipase C/protein kinase C and intracellular calcium (About-Samra, A. B., *et al., Endocrinology 129*:2547-2554 (1991); Jüppner, H., *et al., Science 254*:1024-1026 (1991); Guo, J. *et al., Endocrinology 136*:3884-3891 (1995); Hruska, K.A., *et al., J. Clin. Invest. 79*:230-239 (1987); Donahue, H.J., *et al., J. Biol. Chem. 263*:13522-13527 (1988)). The PTH-1 receptor is a member of the family B subgroup of G protein-coupled receptors, which also includes the receptors for calcitonin and secretin (Kolakowski, L. F., "GCRDb: A G-Protein-Coupled Receptor Database," *Receptors and Channels 2*:1-7 (1994)). Mutagenesis and crosslinking studies have indicated that multiple domains of these receptors contribute to ligand interaction, including the large amino-terminal extracellular domain, the extracellular loops and the transmembrane helices (Jüppner, H., *et al., Endocrinology 134*:879-884 (1994); Lee, C., *et al., Mol. Endo. 9*:1269-1278 (1995); Turner, P., *et al., J. Bone Min. Res. 12(1)*:Abstract 121 (1997); Dautzenberg, F., *et al., Proc. Natl. Acad. Sci. 95*:4941-4946 (1998); Holtmann, M., *et al., J. Biol. Chem. 270*:14394-14398 (1995); DeAlmeida, V. and Mayo, K., *Mol. Endo. 12*:750-765 (1998); Stroop, S., *et al., Biochem. 34*:1050-1057 (1994); Zhou, A., *et al., Proc. Natl. Acad. Sci. USA 94*:3644-

3649 (1997); Bisello, A., *et al., J. Biol. Chem. 273*:22498-22505 (1998)). Studies using PTH/calcitonin chimeric receptors and hybrid ligands have suggested a general topology of the interaction in which the amino-terminal extracellular domain of the receptor recognizes the carboxyl-terminal binding domain of the ligand, while the "core" region of the receptor containing the seven transmembrane helices and connecting loops recognizes the amino-terminal signaling portion of the ligand (Bergwitz, C., *et al., J. Biol. Chem. 271*:26469-26472 (1996)). Similar conclusions were derived from earlier receptor chimera studies (Jüppner, H., *et al., Endocrinology 134*:879-884 (1994); Stroop, S., *et al., Biochem. 34*:1050-1057 (1994); Gardella, T.J., *et al., Endocrinology 135*:1186-1194 (1994)) and from recent crosslinking studies with photoreactive PTH analogs (Bisello, A., *et al., J. Biol. Chem. 273*:22498-22505 (1998); Mannstadt, M., *et al., J. Biol. Chem. 273*:16890-16896 (1998)).

[0006] In the current study we investigate the signaling component of the interaction between PTH and the PTH-1 receptor using a domain-based approach. This approach employs short amino-terminal PTH fragment analogs and a PTH receptor mutant that lacks most of the amino-terminal extracellular domain. The results of cAMP-signaling assays performed with these smaller ligands and receptors demonstrate that the conserved amino-terminal (Kronenberg, H.M., *et al.*, in "Handbook of Experimental Pharmacology, Springer-Verlag," Heidelberg (1993); Lanske, B., *et al., Science 273*:663-666 (1996); Dempster, D.W., *et al., Endocr Rev. 14(6)*:690-709 (1994); Nussbaum, S.R., *et al., J. Biol. Chem. 255*:10183-10187 (1980); Gardella, T. J., *et al., Endocrinology 132(5)*:2024-2030 (1993); M. P. Caulfield *et al., Endocrinology 127*:83-87 (1990); A. B. Abou-Samra *et al., Endocrinology 125*:2215-2217 (1989); Suva, L.J., *et al., Science 237(4817)*:893-896 (1987); Horiuchi, N., *et al., Science 220*:1053-1055 (1983); Nutt, R. F., *et al., Endocrinology 127*:491-493 (1990); Rosenblatt, M., *Pathobilogy Annual*, Raven Press, New York, 11:53-84 (1981); Azarani, A., *et al., J. Biol. Chem. 271(25)*:14931-14936 (1996); Tregear, G.W., *et al., Endocrinology 93*:1349-1353 (1973); About-Samra, A.B., *et al., Endocrinology 129*:2547-2554 (1991)) segment of PTH functions as an autonomous signaling domain and that this domain interacts with the core region of the receptor.

SUMMARY OF THE INVENTION

[0007] PTH is the principal regulator of blood calcium levels and mediates this action through binding to PTH-1 receptors on bone and kidney cells. PTH-1 receptor agonists may ultimately be used to treat metabolic bone diseases, such as osteoporosis. Thus there is a strong need in the art develop new and improved PTH and PTH receptor reagents for the treatment of human disease.

[0008] In a first embodiment, the invention provides a novel PTH-1 receptor polypeptide, rΔNt [[rδNt]], characterized by a deletion of the extracellular amino-terminal ligand binding domain. The invention also provides nucleic acid molecules encoding the rΔNt [[rδNt]] receptor polypeptide.

[0009] In a second embodiment the rΔNt [[rδNt]] receptor of the invention is useful for screening procedures designed to identify agonists and antagonists of PTH receptor function. The invention provides screening utilizing either cAMP accumulation or competitive binding for the evaluation of test compounds with cells expressing the rΔNt [[rδNt]] receptor.

BRIEF DESCRIPTION OF THE FIGURES

[0010] Figure 1. Presentation of the nucleic acid sequence (SEQ ID NO:1) and the amino acid sequence (SEQ ID NO:2) of the mutant PTH1R receptor, rΔNt [[rδNt]].

[0011] Figure 2. cAMP-stimulating Activity of PTH Fragments in LLC-PK1 Cells. A) Rat PTH(1-34) analog or amino-terminal rPTH fragments were tested for cAMP-stimulating activity in an LLC-P1-derived cell line (HKRK-B7) stably transfected with the human PTH-1 receptor. Cells were treated with peptide at the indicated doses for 60 min at 22°C. Intracellular cAMP was measured by RIA, as described in Experimental Procedures. Shown are combined data (mean ± s.e.m.) from 3 separate experiments, each performed in duplicate. B) HKRK-B7 cells, or untransfected LLC-PK1 were treated with rPTH(1-34) or rPTH(1-14), and intracellular cAMP was measured. Shown are data (mean ± s.e.m.) from a single representative experiment performed in duplicate.

[0012] Figure 3. Alanine-scan of PTH(1-14). HKRK-B7 cells were treated with 100 mM of one of 14 different rPTH(1-14) analogs, each having a different alanine-substitution at the indicated amino acid position. The resulting cAMP levels were determined as described in Experimental Procedures. Shown are the combined data (mean ± s.e.m.) from three separate experiments, each performed in duplicate. The mean (mean ±s.e.m.) basal cAMP levels observed in the three experiments was 2. 1 ±0.1 pmole/well, and the maximum response to rPTH(1-34) at 0.1 mM was 254± 16 pmole/well.

[0013] Figure 4. PTH Responses of Intact and Truncated PTH-1 Receptors in COS-7 Cells. Shown at the top are schematics of the intact (A) and truncated (B) rat PTH-1 receptors used for transient transfection of COS-7 cells, and subsequent cAMP response assays. The conserved extracellular cysteine residues are depicted as open circles and numbered according to sequence position, and the nine amino acids of the epitope tag (HA) in rWT-HA are shaded. The tics at residue 26 and 181 indicate the endpoints of the deletion in rΔNt [[rδNt]]. Based on the predicted signal peptide cleavage site at Ala-22, residues 23-25 in rΔNt [[rδNt]] are joined to residue 182. The cAMP responses of COS-7 cells expressing the intact receptor (C) and rΔNt [[rδNt]] (D) to rPTH(1-34) (J) or rPTH(1-14) (C) are also shown. The graphs show combined data (mean ±s.e.m.) from five separate experiments, each performed in duplicate.

[0014] Figure 5. Alanine-scan of PTH(1-14) with Intact and Truncated PTH Receptors. COS-7 cells transiently transfected with rWT-HA (A) or rCE I -G (B) were treated with 100 mM of native rat PTH(1-14) or 100 mM of a rPTH(1-14) analog containing a single alanine substitution for 1h at 21°C, and the resulting intracellular cAMP levels were measured by RIA. The amino acid substitutions are indicated on the axis labels. Peptides were tested in duplicate, and a single experiment representative of three others is shown.

[0015] Figure 6. Specificity of the Truncated Ligand and PTH Receptor. COS-7 cells transiently transfected with either rWT-HA (A), rΔNt [[rδNt]] (B), or the intact rat secretin receptor (C), were treated with the indicated peptides for 60 min at 22°C, and the resulting intracellular cAMP levels were quantified by RIA. Concentration of peptides present during the incubations

were: rPTH(1-34), 0.1 mM; rPTH(1-14) 100 mM; secretin(1-27). 1 mM and secretin(1-13), 100 mM. Shown are data (mean ± s.e.m.) from one experiment performed in duplicate, and this was repeated twice more with equivalent results.

[0016] Figure 7. Antagonist Properties of PTHrP(7-34) with PTH(1-14). COS-7 cells transfected with rWT-HA (A) or rΔNt [[rδNt]] (B) were treated with the antagonist [Leu11,D-Trp12]hPTHrP(7-34)NH2 (or buffer alone), for 5 min at 22EC, followed by 10 ml of either rPTH(1-34) or rPTH(1-14) agonist peptide. Incubations were continued for 30 min at 21°C and the resulting cAMP levels were measured by RIA, as described in Experimental Procedures. The final concentration of antagonist peptide present during the incubation was 10 mM. Shown are data from a single experiment performed in triplicate. A repeat of the same experiment yielded equivalent results.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

[0017] The present invention provides isolated nucleic acid molecules comprising a polynucleotide encoding the rΔNt [[rδNt]] receptor polypeptide, a novel, mutant PTH1R receptor polypeptide, having the amino acid sequence shown in Figure 1 (SEQ ID NO:2), which was determined by sequencing a cloned cDNA. The rΔNt [[rδNt]] protein of the present invention shares sequence homology with previously identified non-mutant PTH1R and PTH2R sequences. The nucleotide sequence shown in Figure 1 (SEQ ID NO:1) was obtained by sequencing a cDNA clone (rΔNt [[rδNt]]), which was deposited on [[____]] December 28, 1999 at the American Type Culture Collection, 10801 University Boulevard, Manassas, Virginia 20110-2209, and given accession number PTA-1136 [[____]].

1. The rΔNt [[rδNt]] Receptor

a) Nucleic Acid Molecules

[0018] Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined by manual sequencing, and all amino acid sequences of polypeptides encoded by DNA molecules

determined herein were predicted by translation of a DNA sequence determined as above. Therefore, as is known in the art for any DNA sequence determined by this approach, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by manual sequencing are typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. As is also known in the art, a single insertion or deletion in a determined nucleotide sequence compared to the actual sequence will cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely different from the amino acid sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

[0019] Using the information provided herein, such as the nucleotide sequence in Figures 1, a nucleic acid molecule of the present invention encoding a rΔNt [[rδNt]] polypeptide, respectively, may be obtained using standard techniques. Cloning and screening procedures are known for the isolation of the wild-type PTH1R sequence, such as those for cloning cDNAs using mRNA as starting material. Subsequent to cloning the wild-type receptor, the appropriate deletion in the sequence may be made as described herein. Illustrative of the invention, the nucleic acid molecule described in Figure 1 (SEQ ID NO:1) was obtained by using standard restriction enzyme digestion and cloning techniques in the art. The determined nucleotide sequence of the rΔNt [[rδNt]] cDNA of Figure 1 (SEQ ID NO:1) contains an open reading frame encoding a protein of about 435 amino acid residues, with a predicted leader sequence of about 22 amino acid residues. The amino acid sequence of the predicted mature rΔNt [[rδNt]] receptor is shown in Figure 1 from amino acid residue about 23 to residue about 435. The rΔNt [[rδNt]] protein shown in Figure 1 (SEQ ID NO:2) is about 84% identical to the rat PTH1 receptor.

[0020] As indicated, the present invention also provides the mature form(s) of the rΔNt [[rδNt]] receptor of the present invention. According to the signal hypothesis, proteins secreted by mammalian cells have a signal or secretory leader sequence which is cleaved from the mature protein once export of the growing protein chain across the rough endoplasmic reticulum has been initiated. Most mammalian cells and even insect cells cleave secreted proteins

with the same specificity. However, in some cases, cleavage of a secreted protein is not entirely uniform, which results in two or more mature species on the protein. Further, it has long been known that the cleavage specificity of a secreted protein is ultimately determined by the primary structure of the complete protein, that is, it is inherent in the amino acid sequence of the polypeptide. Therefore, the present invention provides a nucleotide sequence encoding the mature rΔNt [[rδNt]] polypeptides having the amino acid sequence encoded by the cDNA clone contained in the host identified as ATCC Deposit No. PTA-1136 [[____]] and as shown in Figure 1 (SEQ ID NO:2). By the mature rΔNt [[rδNt]] protein having the amino acid sequence encoded by the cDNA clone contained in the host identified as ATCC Deposit PTA-1136 [[____]] is meant the mature form(s) of the rΔNt [[rδNt]] receptor produced by expression in a mammalian cell (e.g., COS cells, as described below) of the complete open reading frame encoded by the DNA sequence of the clone contained in the vector in the deposited host. As indicated below, the mature rΔNt [[rδNt]] receptor having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. PTA-1136 [[____]] may or may not differ from the predicted "mature"rΔNt [[rδNt]] protein shown in Figure 1 (amino acids from about 23 to about 435) depending on the accuracy of the predicted cleavage.

[0021] Methods for predicting whether a protein has a secretory leader as well as the cleavage point for that leader sequence are available. For instance, the methods of McGeoch (*Virus Res. 3*:271-286 (1985)) and von Heinje (*Nucleic Acids Res. 14*:4683-4690 (1986)) can be used. The accuracy of predicting the cleavage points of known mammalian secretory proteins for each of these methods is in the range of 75-80%. von Heinje, *supra.* However, the two methods do not always produce the same predicted cleavage point(s) for a given protein. A computational method may be found in the computer program "PSORT" (K. Nakai and M. Kanehisa, Genomics 14:897-911 (1992)), which is an expert system for predicting the cellular location of a protein based on the amino acid sequence. As part of this computational prediction of localization, the methods of McGeoch and von Heinje are incorporated.

[0022] In the present case, the predicted amino acid sequence of the complete rΔNt [[rδNt]] polypeptide of the present invention was analyzed for structural properties by comparison to the rat rΔNt [[rδNt]] sequence. This analysis provided predicted a cleavage site between amino acids 22 and 23 in Figure 1 (SEQ ID NO:2). Thus, the leader sequence for the rΔNt [[rδNt]] receptor protein is predicted to consist of amino acid residues 1-22 in Figure 1 (amino acids 1 to 22 in SEQ ID NO:2) , while the predicted mature rΔNt [[rδNt]] protein consists of residues 23-435 (amino acids 23 to 435 in SEQ ID NO:2).

[0023] As indicated, nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

[0024] As one of ordinary skill would appreciate, however, due to the possibilities of sequencing errors, the rΔNt [[rδNt]] receptor polypeptide encoded by the deposited cDNA comprises about 435 amino acids, but may be anywhere in the range of 425-435 amino acids; and the leader sequence of this protein is about 22 amino acids, but may be anywhere in the range of about 10 to about 30 amino acids.

[0025] As indicated, nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

[0026] By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or

substantially) DNA molecules in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

[0027] Isolated nucleic acid molecules of the present invention include DNA molecules comprising an open reading frame (ORF) shown in Figure 1 (SEQ ID NO:1); DNA molecules comprising the coding sequence for the rΔNt [[rδNt]] receptor shown in Figure 1 (SEQ ID NO:2); and DNA molecules which comprise a sequence substantially different from those described above but which, due to the degeneracy of the genetic code, still encode the rΔNt [[rδNt]] receptor. Of course, the genetic code is well known in the art. Thus, it would be routine for one skilled in the art to generate such degenerate variants.

[0028] In another aspect, the invention provides isolated nucleic acid molecules encoding the rΔNt [[rδNt]] polypeptide having an amino acid sequence encoded by the cDNA clone contained in the plasmid deposited as ATCC Deposit No. PTA-1136 [[____]] on December 28, 1999 [[____]]. Preferably, the nucleic acid molecule will encode the mature polypeptide encoded by the above-described deposited cDNA clone. In a further embodiment, a nucleic acid molecule is provided encoding the rΔNt [[rδNt]] polypeptide or the rΔNt [[rδNt]] polypeptide lacking the N-terminal methionine. The invention also provides an isolated nucleic acid molecule having the nucleotide sequence shown in SEQ ID NO:1 or the nucleotide sequence of the rΔNt [[rδNt]] cDNA contained in the above-described deposited clone, or a nucleic acid molecule having a sequence complementary to one of the above sequences. Such isolated molecules, particularly DNA molecules, are useful as probes for gene mapping, by *in situ* hybridization with chromosomes, and for detecting expression of the rΔNt [[rδNt]] gene in human tissue, for instance, by Northern blot analysis.

[0029] The present invention is further directed to fragments of the isolated nucleic acid molecules described herein. By a fragment of an isolated nucleic acid molecule having the nucleotide sequence of the deposited cDNAs or the nucleotide sequence shown in Figure 1 (SEQ ID NO:1) is intended fragments at least about 15 nt, and more preferably at least about 20 nt, still more

preferably at least about 30 nt, and even more preferably, at least about 40 nt in length which are useful as diagnostic probes and primers as discussed herein. Of course, larger fragments of about 50-1550 nt in length, and more preferably at fragments least about 600 nt in length are also useful according to the present invention as are fragments corresponding to most, if not all, of the nucleotide sequence of the deposited cDNAs or as shown in Figure 1 (SEQ ID NO:1). By a fragment at least 20 nt in length, for example, is intended fragments which include 20 or more contiguous bases from the nucleotide sequence of the deposited cDNAs or the nucleotide sequence as shown in Figure 1 (SEQ ID NO:1).

[0030] Preferred nucleic acid fragments of the present invention include nucleic acid molecules encoding: a polypeptide comprising the rΔNt [[rδNt]] receptor extracellular domain (predicted to constitute amino acid residues from about 23 to about 147 in Figure 1 (or amino acid residues from about 23 to about 147 in SEQ ID NO:2)); a polypeptide comprising the rΔNt [[rδNt]] receptor transmembrane domain (predicted to constitute amino acid residues from about 148 to about 416 in Figure 1 (or amino acid residues from about 148 to about 416 in SEQ ID NO:2)); and a polypeptide comprising the rΔNt [[rδNt]] receptor extracellular domain with all or part of the transmembrane domain deleted. As above with the leader sequence, the amino acid residues constituting the rΔNt [[rδNt]] receptor extracellular and transmembrane domains have been predicted. Thus, as one of ordinary skill would appreciate, the amino acid residues constituting these domains may vary slightly (e.g., by about 1 to about 15 amino acid residues) depending on the criteria used to define each domain.

[0031] Preferred nucleic acid fragments of the present invention also include nucleic acid molecules encoding epitope-bearing portions of the rΔNt [[rδNt]] receptor protein. As one skilled in the art would know, a nucleic acid sequence may be used to predict the polypeptide sequence encoded therein. Such information may then be used to predict antigenic determinants in the polypeptide that may be related to the corresponding polynucleotide regions encoding the antigenic determinants identified by the analysis. Methods for predicting the antigenic determinants of a polypeptide are well known in the art.

[0032] Methods for determining other such epitope-bearing portions of the rΔNt [[rδNt]] protein are described in detail below.

[0033] In another aspect, the invention provides an isolated nucleic acid molecule comprising a polynucleotide which hybridizes under stringent hybridization conditions to a portion of the polynucleotide in a nucleic acid molecule of the invention described above, for instance, the cDNA clones contained in ATCC Deposit Nos. PTA-1136, PTA-1138, PTA-1139, PTA-1140, PTA-1142, PTA-1137 or PTA-1141[[_____ or _____]]. By "stringent hybridization conditions" is intended overnight incubation at 42°C in a solution comprising: 50% formamide, 5x SSC (150 mM NaCl, 15mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 g/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65°C.

[0034] By a polynucleotide which hybridizes to a "portion" of a polynucleotide is intended a polynucleotide (either DNA or RNA) hybridizing to at least about 15 nucleotides (nt), and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably about 30-70 nt of the reference polynucleotide. These are useful as diagnostic probes and primers as discussed above and in more detail below.

[0035] By a portion of a polynucleotide of "at least 20 nt in length," for example, is intended 20 or more contiguous nucleotides from the nucleotide sequence of the reference polynucleotide (e.g., the deposited cDNAs or the nucleotide sequence as shown in Figure 1 (SEQ ID NO:1).

[0036] Of course, a polynucleotide which hybridizes only to a poly A sequence (such as the 3N terminal poly(A) tract of the rΔNt [[rδNt]] receptor cDNA shown in Figure 1 (SEQ ID NO:1), or to a complementary stretch of T (or U) resides, would not be included in a polynucleotide of the invention used to hybridize to a portion of a nucleic acid of the invention, since such a polynucleotide would hybridize to any nucleic acid molecule containing a poly (A) stretch or the complement thereof (e.g., practically any double-stranded cDNA clone).

[0037] As indicated, nucleic acid molecules of the present invention which encode a rΔNt [[rδNt]] polypeptide may include, but are not limited to those

encoding the amino acid sequence of the mature polypeptides, by themselves; the coding sequence for the mature polypeptides and additional sequences, such as those encoding the amino acid leader or secretory sequence, such as a pre-, or pro- or prepro- protein sequence; the coding sequence of the mature polypeptide, with or without the aforementioned additional coding sequences, together with additional, non-coding sequences, including for example, but not limited to introns and non-coding 5N and 3N sequences, such as the transcribed, non-translated sequences that play a role in transcription, mRNA processing, including splicing and polyadenylation signals, for example - ribosome binding and stability of mRNA; an additional coding sequence which codes for additional amino acids, such as those which provide additional functionalities. Thus, the sequence encoding the polypeptide may be fused to a marker sequence, such as a sequence encoding a peptide which facilitates purification of the fused polypeptide. In certain preferred embodiments of this aspect of the invention, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described in Gentz *et al., Proc. Natl. Acad. Sci. USA 86*:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. The "HA" tag is another peptide useful for purification which corresponds to an epitope derived from the influenza hemagglutinin protein, which has been described by Wilson *et al., Cell 37*: 767 (1984). As discussed below, other such fusion proteins include the rΔNt [[rδNt]] receptor fused to Fc at the amino or C-terminus.

[0038] The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of the rΔNt [[rδNt]] receptor. Variants may occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. *Genes II*, Lewin, B., ed., John Wiley & Sons, New York (1985). Non-naturally occurring variants may be produced using art-known mutagenesis techniques.

[0039] Such variants include those produced by nucleotide substitutions, deletions or additions, which may involve one or more nucleotides. The variants may be altered in coding regions, non-coding regions, or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the rΔNt [[rδNt]] receptor or portions thereof. Also especially preferred in this regard are conservative substitutions.

[0040] Further embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 95%, 96%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding the full-length rΔNt [[rδNt]] polypeptide having the complete amino acid sequence in SEQ ID NO:2, including the predicted leader sequence; (b) a nucleotide sequence encoding the polypeptide having the amino acid sequence in SEQ ID NO:2, but lacking the N-terminal methionine; (c) a nucleotide sequence encoding the mature rΔNt [[rδNt]] receptor (full-length polypeptide with the leader removed) having the amino acid sequence at positions from about 23 to about 435 in SEQ ID NO:2; (d) a nucleotide sequence encoding the full-length rΔNt [[rδNt]] polypeptide having the complete amino acid sequence including the leader encoded by the cDNA clone contained in ATCC Deposit No. PTA-1136 [[____]]; (e) a nucleotide sequence encoding the mature rΔNt [[rδNt]] receptor having the amino acid sequence encoded by the cDNA clone contained in ATCC Deposit No. 97883; (f) a nucleotide sequence encoding the rΔNt [[rδNt]] receptor extracellular domain; (g) a nucleotide sequence encoding the rΔNt [[rδNt]] receptor transmembrane domain; (h) a nucleotide sequence encoding the rΔNt [[rδNt]] receptor extracellular domain with all or part of the transmembrane domain deleted; and (i) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), (c), (d), (e), (f), (g) or (h).

[0041] By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence encoding a rΔNt [[rδNt]] polypeptide is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the

polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the rΔNt [[rδNt]] receptor. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% of the nucleotides in the reference sequence may be deleted or substituted with another nucleotide, or a number of nucleotides up to 5% of the total nucleotides in the reference sequence may be inserted into the reference sequence. These mutations of the reference sequence may occur at the 5N or 3N terminal positions of the reference nucleotide sequence or anywhere between those terminal positions, interspersed either individually among nucleotides in the reference sequence or in one or more contiguous groups within the reference sequence.

[0042] As a practical matter, whether any particular nucleic acid molecule is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the nucleotide sequence shown in Figure 1 or to the nucleotides sequence of the deposited cDNA clones can be determined conventionally using known computer programs such as the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711. Bestfit uses the local homology algorithm of Smith and Waterman, *Advances in Applied Mathematics 2*: 482-489 (1981), to find the best segment of homology between two sequences. When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference nucleotide sequence and that gaps in homology of up to 5% of the total number of nucleotides in the reference sequence are allowed.

[0043] The present application is directed to nucleic acid molecules at least 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence shown in Figure 1 (SEQ ID NO:1) or to the nucleic acid sequence of the deposited cDNAs, irrespective of whether they encode a polypeptide having rΔNt [[rδNt]] receptor activity. This is because even where a particular nucleic acid molecule does not encode a polypeptide having rΔNt [[rδNt]] receptor

activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe or a polymerase chain reaction (PCR) primer. Uses of the nucleic acid molecules of the present invention that do not encode a polypeptide having rΔNt [[rδNt]] receptor activity include, *inter alia,* (1) isolating the rΔNt [[rδNt]] receptor gene or allelic variants thereof in a cDNA library; (2) *in situ* hybridization (e.g., "FISH") to metaphase chromosomal spreads to provide precise chromosomal location of the rΔNt [[rδNt]] receptor gene, as described in Verma *et al., Human Chromosomes: A Manual of Basic Techniques*, Pergamon Press, New York (1988); and (3) Northern Blot analysis for detecting rΔNt [[rδNt]] receptor mRNA expression in specific tissues.

[0044] Preferred, however, are nucleic acid molecules having sequences at least 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence shown in Figure 1 (SEQ ID NO:1) or to the nucleic acid sequence of the deposited cDNA which do, in fact, encode a polypeptide having rΔNt [[rδNt]] receptor activity. By "a polypeptide having rΔNt [[rδNt]] receptor activity" is intended polypeptides exhibiting activity similar, but not necessarily identical, to an activity of the rΔNt [[rδNt]] receptor of the invention, as measured in a particular biological assay. For example, rΔNt [[rδNt]] receptor activity can be measured using competition binding experiments of labeled PTH or PTHrP to cells expressing the candidate rΔNt [[rδNt]] polypeptide as described herein.

[0045] Any cell line expressing the rΔNt [[rδNt]] receptor, or variants thereof, may be used to assay ligand binding and second messenger activation as described in Examples 3 and 4. Of course, due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least 95%, 96%, 97%, 98%, or 99% identical to the nucleic acid sequence of the deposited cDNAs or the nucleic acid sequence shown in Figure 1 (SEQ ID NO:1) will encode a polypeptide "having rΔNt [[rδNt]] receptor activity." In fact, since degenerate variants of these nucleotide sequences all encode the same polypeptide, this will be clear to the skilled artisan even without performing the above described comparison assay. It will be further recognized in the art that, for such nucleic acid molecules that are not

degenerate variants, a reasonable number will also encode a polypeptide having rΔNt [[rδNt]] protein activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein function (e.g., replacing one aliphatic amino acid with a second aliphatic amino acid).

[0046] For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided in Bowie, J. U. *et al.,* "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science 247:*1306-1310 (1990), wherein the authors indicate that proteins are surprisingly tolerant of amino acid substitutions.

b) Vectors and Host Cells

[0047] The present invention also relates to vectors which include the isolated DNA molecules of the present invention, host cells which are genetically engineered with the recombinant vectors, and the production of rΔNt [[rδNt]] polypeptides or fragments thereof by recombinant techniques.

[0048] The polynucleotides may be joined to a vector containing a selectable marker for propagation in a host. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. If the vector is a virus, it may be packaged *in vitro* using an appropriate packaging cell line and then transduced into host cells.

[0049] The DNA insert should be operatively linked to an appropriate promoter, such as the phage lambda PL promoter, the *E. coli lac, trp* and *tac* promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name a few. Other suitable promoters will be known to the skilled artisan. The expression constructs will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will preferably include a translation initiating at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

[0050] As indicated, the expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture and tetracycline or ampicillin resistance

genes for culturing in *E. coli* and other bacteria. Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS and Bowes melanoma cells; and plant cells. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

[0051] Among vectors preferred for use in bacteria include pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan.

[0052] Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis *et al., Basic Methods In Molecular Biology* (1986).

[0053] The polypeptide may be expressed in a modified form, such as a fusion protein, and may include not only secretion signals, but also additional heterologous functional regions. For instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification, or during subsequent handling and storage. Also, peptide moieties may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and to facilitate purification, among others, are familiar and routine techniques in the art. A preferred fusion protein comprises a heterologous region from immunoglobulin that is useful to solubilize proteins. For example, EP-A-O 464 533 (Canadian counterpart 2045869) discloses fusion proteins comprising various portions of constant

region of immunoglobin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is thoroughly advantageous for use in therapy and diagnosis and thus results, for example, in improved pharmacokinetic properties (EP-A 0232 262). On the other hand, for some uses it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified in the advantageous manner described. This is the case when Fc portion proves to be a hindrance to use in therapy and diagnosis, for example when the fusion protein is to be used as antigen for immunizations. In drug discovery, for example, human proteins, such as, hIL5-receptor has been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. See, D. Bennett *et al.*, *Journal of Molecular Recognition*, Vol. 8:52-58 (1995) and K. Johanson *et al.*, *The Journal of Biological Chemistry*, Vol. 270, No. 16:9459-9471 (1995).

[0054] The rΔNt [[rδNt]] receptor can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification. Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

c) rΔNt [[rδNt]] Polypeptides and Fragments

[0055] The invention further provides an isolated rΔNt [[rδNt]] polypeptide having the amino acid sequence encoded by the deposited cDNAs, or the

amino acid sequence in Figure 1 (SEQ ID NO:2) or a peptide or polypeptide comprising a portion of the above polypeptides.

[0056] It will be recognized in the art that some amino acid sequences of the rΔNt [[rδNt]] receptor can be varied without significant effect of the structure or function of the protein. If such differences in sequence are contemplated, it should be remembered that there will be critical areas on the protein which determine activity. Thus, the invention further includes variations of the rΔNt [[rδNt]] receptor which show substantial rΔNt [[rδNt]] receptor activity or which include regions of rΔNt [[rδNt]] protein such as the protein portions discussed below. Such mutants include deletions, insertions, inversions, repeats, and type substitutions. As indicated above, guidance concerning which amino acid changes are likely to be phenotypically silent can be found in Bowie, J.U., *et al.,* "Deciphering the Message in Protein Sequences: Tolerance to Amino Acid Substitutions," *Science 247:*1306-1310 (1990).

[0057] Thus, the fragment, derivative or analog of the polypeptide of Figure 1 (SEQ ID NO:2) or that encoded by the deposited cDNAs, may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as an IgG Fc fusion region peptide or leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

[0058] Of particular interest are substitutions of charged amino acids with another charged amino acid and with neutral or negatively charged amino acids. The latter results in proteins with reduced positive charge to improve the characteristics of the rΔNt [[rδNt]] protein. The prevention of aggregation is highly desirable. Aggregation of proteins not only results in a loss of activity but can also be problematic when preparing pharmaceutical

formulations, because they can be immunogenic. (Pinckard *et al., Clin Exp. Immunol. 2*:331-340 (1967); Robbins *et al., Diabetes 36*:838-845 (1987); Cleland *et al. Crit. Rev. Therapeutic Drug Carrier Systems 10*:307-377 (1993)).

[0059] The replacement of amino acids can also change the selectivity of binding to cell surface receptors. Ostade *et al., Nature 361*:266-268 (1993) describes certain mutations resulting in selective binding of TNF-α to only one of the two known types of TNF receptors. Thus, the rΔNt [[rδNt]] receptor of the present invention may include one or more amino acid substitutions, deletions or additions, either from natural mutations or human manipulation.

[0060] As indicated, changes are preferably of a minor nature, such as conservative amino acid substitutions that do not significantly affect the folding or activity of the protein (see Table 1).

TABLE 1. Conservative Amino Acid Substitutions.

Aromatic	Phenylalanine Tryptophan Tyrosine
Hydrophobic	Leucine Isoleucine Valine
Polar	Glutamine Asparagine
Basic	Arginine Lysine Histidine
Acidic	Aspartic Acid Glutamic Acid
Small	Alanine Serine Threonine Methionine Glycine

[0061] Amino acids in the rΔNt [[rδNt]] protein of the present invention that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (Cunningham

and Wells, *Science 244*:1081-1085 (1989)). The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for biological activity such as receptor binding or *in vitro* proliferative activity. Sites that are critical for ligand-receptor binding can also be determined by structural analysis such as crystallization, nuclear magnetic resonance or photoaffinity labeling (Smith *et al., J. Mol. Biol. 224*:899-904 (1992) and de Vos *et al. Science 255*:306-312 (1992)).

[0062] The polypeptides of the present invention are preferably provided in an isolated form. By "isolated polypeptide" is intended a polypeptide removed from its native environment. Thus, a polypeptide produced and/or contained within a recombinant host cell is considered isolated for purposes of the present invention. Also intended as an "isolated polypeptide" are polypeptides that have been purified, partially or substantially, from a recombinant host cell. For example, a recombinantly produced version of the antimicrobial peptide polypeptide can be substantially purified by the one-step method described in Smith and Johnson, *Gene 67:*31-40 (1988).

[0063] The polypeptides of the present invention are preferably provided in an isolated form, and preferably are substantially purified. A recombinantly produced version of the rΔNt [[rδNt]] receptor can be substantially purified by the one-step method described in Smith and Johnson, *Gene 67:*31-40 (1988).

[0064] The polypeptides of the present invention also include the polypeptide encoded by the deposited rΔNt [[rδNt]] cDNA including the leader, the polypeptide encoded by the deposited the cDNA minus the leader (i.e., the mature protein), the polypeptide of Figure 1 (SEQ ID NO:2) including the leader, the polypeptide of Figure 1 (SEQ ID NO:2) minus the leader, the extracellular domain, the transmembrane domain, a polypeptide comprising amino acids about 1 to about 435 in SEQ ID NO:2, and a polypeptide comprising amino acids about 2 to about 435 in SEQ ID NO:2, as well as polypeptides which are at least 95% identical, still more preferably at least 96%, 97%, 98% or 99% identical to the polypeptides described above, and also include portions of such polypeptides with at least 30 amino acids and more preferably at least 50 amino acids.

[0065] By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a reference amino acid sequence of a rΔNt [[rδNt]] polypeptide is intended that the amino acid sequence of the polypeptide is identical to the reference sequence except that the polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the reference amino acid of the rΔNt [[rδNt]] receptor. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a reference amino acid sequence, up to 5% of the amino acid residues in the reference sequence may be deleted or substituted with another amino acid, or a number of amino acids up to 5% of the total amino acid residues in the reference sequence may be inserted into the reference sequence. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

[0066] As a practical matter, whether any particular polypeptide is at least 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequence shown in Figure 1 (SEQ ID NO:2) to the amino acid sequence encoded by deposited cDNA clones can be determined conventionally using known computer programs such the Bestfit program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, 575 Science Drive, Madison, WI 53711). When using Bestfit or any other sequence alignment program to determine whether a particular sequence is, for instance, 95% identical to a reference sequence according to the present invention, the parameters are set, of course, such that the percentage of identity is calculated over the full length of the reference amino acid sequence and that gaps in homology of up to 5% of the total number of amino acid residues in the reference sequence are allowed.

2. Agonists and Antagonists of the rΔNt [[rδNt]] Receptor Activity

[0067] Functional characterization of the biological properties of the rΔNt [[rδNt]] receptor of the invention and derivatives thereof may be performed by bioassays that measure ligand-stimulated cAMP accumulation.

A. Assay for the Detection of Cyclic AMP Accumulation in Cells Expressing rΔNt [[rδNt]] Receptor After Exposure to Test Compounds

[0068] Intracellular cAMP accumulation is measured as described previously (Abou-Samra *et al., J. Biol. Chem. 262*:1129, 1986). Cells expressing the rΔNt [[rδNt]] receptor grown in 24-well plates are rinsed with culture medium containing 0.1% BSA and 2 mM IBMX. The cells are then incubated with a test compound for 60 min. at 21°C. The supernatant is removed and the cells immediately frozen by placing the whole plate in dry ice powder. Intracellular cAMP is extracted by thawing the cells in 1 ml of 50 mM HCl and analyzed by a specific radioimmunoassay using an anti-cAMP antibody (e.g., Sigma, St. Louis, Mo). A cAMP analog (2'-O-monosuccinyl-adenosine 3':5'-cyclic monophosphate tyrosyl methyl ester, obtained from Sigma) which is used a tracer for cAMP is iodinated by the chloramine T method. Free iodine is removed by adsorbing the iodinated cAMP analog onto a C18 SEP-PAK ~~Sep-pak~~ cartridge (Waters, Milford, Mass.). After washing with dH_20, the iodinated cAMP analog is eluted from the SEP-PAK ~~Sep-pak~~ Cartridge with 40% acetonitrille (ACN) and 0.1% trifluoroacetic acid (TFA). The iodinated cAMP analog is lyophilized, reconstituted in 1 ml 0.1% TFA, and injected into a C18 reverse phase HPLC column (Waters). The column is equilibrated with 10% ACN in 0.1% TFA, and eluted with gradient of 10-30% ACN in 0.1% TFA. This allows separation of the mono-iodinated cAMP analog from the non-iodinated cAMP analog. The tracer is stable for up to 4 months when stored at - 20°C. The standard used for the assay, adenosine 3':5'-cyclic monophosphate, may be purchased from Sigma. Samples (1-10 82 l of HCl extracts) or standards (0.04-100 fmol/tube) are diluted in 50 mM Na-acetate (pH 5.5), and acetylated with 10 µl of mixture of triethylamine and acetic anhydride (2:1 vol:vol). After acetylation, cAMP antiserum (100 µl) is added from a stock solution (1:4000) made in PBS (pH 7.4), 5 mM EDTA and 1% normal rabbit serum. The tracer is diluted in PBS (pH 7.4) with 0.1% BSA, and added (20,000 cpm/tube). The assay is incubated at 4°C. overnight. The bound tracer is precipitated by adding 100 µl of goat anti-rabbit antiserum

(1:20 in PBS) and 1 ml of 7% polyethyleneglycol (MW 5000-6000), centrifuging at 2000 rpm for 30 min. at 4°C. The supernatant is removed and the bound radioactivity is counted in a gamma-counter (Micromedic). To compute the cAMP data, logit calculations are performed in Excel spreadsheets. Typically, the assay sensitivity is 0.1 fmol/tube, and the standard concentration that displaces 50% of tracer is 5 fmol/tube.

B. Screening Compounds Utilizing an rΔNt [[rδNt]] Receptor Binding Assay

[0069] In addition to the cAMP accumulation assay described below, it is possible that compounds may be iodinated and used in a radioreceptor-based assay in rΔNt [[rδNt]] transiently transfected COS cells. COS-7 cells are grown in 15 cm plates in DMEM, 10% heat-inactivated FBS, 10 mg/L gentamycin until 80-90% confluent. Twenty-four hours after transfection by the DEAE/Dextran method (Sambrook *et al.*, *supra*), with 1-2μg of plasmid DNA, the cells are trypsinized and replated in multiwell plastic dishes (16 or 35 mm diameter, Costar, Cambridge, Mass.) at a cell concentration of 5×10^4 cells/cm^2. Cell number increased only slightly after transfection. After continuing culture for another 48 h, radioreceptor assays are performed. The culture medium is replaced with buffer containing 50 mM Tris-HCL (pH 7.7), 100 mM NaCl, 2 mM $CaCl_2$ 5 mM KCL, 0.5% heat-inactivated fetal bovine serum (GIBCO), and 5% heat-inactived horse serum (KC Biological Inc., Lenexa, Kans.) immediately before studies are initiated. Unless otherwise indicated, studies are conducted with cells incubated in this buffer at 15°C. for 4 h with 4×10^5 cpm/ml (9.6×10^{-11} M) of ^{125}I-labeled [Ala^1]PTH(1-14) amide or ^{125}I-labeled [Nle^8]PTH(1-14).

C. Screening for PTH-1 Receptor Antagonists and Agonists

[0070] The rΔNt [[rδNt]] receptor of the invention may be utilized in to screen for compounds that are agonistic or antagonistic to the PTH response using the cAMP accumulation assay. Cells expressing PTH-1 receptor on the cell surface are incubated with native PTH(1-84) for 5-60 minutes at 37°C., in the presence of 2 mM IBMX (3-isobutyl-1-methyl-xanthine, Sigma, St. Louis,

MO). Cyclic AMP accumulation is measured by specific radio-immunoassay, as described above. A test compound that competes with native PTH(1-84) for binding to the rΔNt [[rδNt]] receptor, and that inhibits the effect of native PTH(1-84) on cAMP accumulation, is considered a competitive antagonist. Such a compound would be useful for treating hypercalcemia.

[0071] Conversely, a test compound that does not compete with native PTH(1-84) for binding to the rΔNt [[rδNt]] receptor, but which still prevents native PTH(1-84) activation of cAMP accumulation (presumably by blocking the receptor activation site) is considered a non-competitive antagonist. Such a compound would be useful for treating hypercalcemia.

[0072] A candidate compound that competes with native PTH(1-84) for binding to the rΔNt [[rδNt]] receptor, and which stimulates cAMP accumulation in the presence or absence of native PTH(1-84) is a competitive agonist. A candidate compound that does not compete with native PTH(1-84) for binding to the rΔNt [[rδNt]] receptor but which is still capable of stimulating cAMP accumulation in the presence or absence of native PTH(1-84), or which stimulates a higher cAMP accumulation than that observed with a PTH compound would be considered a non-competitive agonist.

[0073] Thus, in a further aspect, a screening method is provided for determining whether a candidate agonist or antagonist is capable of enhancing or inhibiting a cellular response to PTH or PTHrP. The method involves contacting cells which express the rΔNt [[rδNt]] polypeptide with a candidate compound and the PTH or PTHrP ligand, assaying a cellular response, and comparing the cellular response to a standard cellular response, the standard being assayed when contact is made with the ligand in absence of the candidate compound, whereby an increased cellular response over the standard indicates that the candidate compound is an agonist of the ligand/receptor signaling pathway and a decreased cellular response compared to the standard indicates that the candidate compound is an antagonist of the ligand/receptor signaling pathway. By "assaying a cellular response" is intended qualitatively or quantitatively measuring a cellular response to a candidate compound and/or PTH or PTHrP (e.g., cyclic AMP accumulation). By the invention, a cell expressing the rΔNt [[rδNt]] polypeptide can be contacted with either an endogenous or exogenously administered PTH or PTHrP.

EXAMPLES

General Methods

[0074] ***Peptides:*** Peptides were prepared by the Biopolymer Synthesis Facility at Massachusetts General Hospital (Boston, MA) using solid-phase chemistry with Fmoc (N-(9-fluorenyl)methoxycarbonyl) protecting groups, and TFA-mediated cleavage and deprotection. All peptides were C-terminally amidated. The PTH(1-14) analogs were synthesized on a multiple peptide synthesizer (Advanced Chemtech Model 396 MBS) at 0.025 mM scale. The completed peptides were desalted by adsorption on a C18 cartridge (Sep-Pak) and then analyzed by reversed-phase C18-based HPLC, MALDI-mass spectromety and amino acid analysis. The PTH(1-34) control peptide, $[Nle^{8,21},Tyr^{34}]rPTH\text{-}(1\text{-}34)NH_2$, and the PTHrP(7-34) antagonist peptide, $[Leu^{11},D\text{-}Trp^{12}]hPTHrP(7\text{-}34)NH_2$, were prepared on an Applied Biosystems Synthesizer (Model 431A) 0.1 mM scale, purified by reversed-phase C18-based HPLC and characterized as described above. Concentrated stock solutions of peptides, 10 mM for PTH(1-14) analogs and 0.3 mM for PTHrP(7-34) and PTH(1-34), were prepared in 10 mM acetic acid, quantified by acid hydrolysis and amino acid analysis and stored at -80°C.

[0075] ***Cell Culture and DNA Transfection:*** COS-7 and HKRK-B7 cells were cultured at 37°C in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (10%); penicillin G (20 units/ml), streptomycin sulfate (20 μg/ml) and amphotericin B (0.05 μg/ml) in a humidified atmosphere containing 5% CO_2. Twenty-four to 16 hours prior to assay, cells in 24-well plates were shifted to a humidified incubator containing 5% CO_2 that was set at 33°C. Stock solutions of EGTA/trypsin and antibiotics were from GIBCO; fetal bovine serum was from Hyclone Laboratories (Logan, UT). Derivation and characterization of the HKRK-B7 cell line by stable transfection of LLC-PK_1 cells with a pCDNA-1-based plasmid (In Vitrogen, San Diego, CA) encoding the hPTH-1 receptor was described previously (Takasu, H. and Bringhurst, F., *Endocrinology*, in press (1998)). These cells express PTH-1 receptors at a surface density of about $1x10^6$ PTH-

binding sites per cell. The HKRK-B7 cells were used for functional assays 24 to 72 hours after the cell monolayer reached confluency.

[0076] For studies with the intact and truncated rat PTH-1 receptors, transient transfections of COS-7 cells were performed using DEAE-dextran as described previously (Bergwitz, C., *et al.*, *J. Biol. Chem. 272*:28861-28868)). The construction and initial characterization of the pCDNA-1 based plasmids encodin either the intact or truncated rat PTH-1 receptor has been described previously (Lee, C., *et al.*, *Endocrinology 135(4)*:1488-1495)). The intact receptor (rWT-HA) contains a nine amino acid HA epitope tag in place of residues 93-101 of the extracellular domain; this epitope tag does not affect receptor function (Lee, C., *et al.*, *Endocrinology 135(4)*:1488-1495)). The truncated rat PTH-1 receptor (rΔNt [[rδNt]]) is deleted for exons E1 through exon G (residues 26 to 181). Assuming that signal peptidase cleavage occurs between Ala-22 and Tyr-23 (Nielsen, H., *et al.*, *Protein Engineering 10*:1-6 (1997)), rΔNt [[rδNt]] is predicted to have for its N-terminus residues Dautzenberg, F., *et al.*, *Proc. Natl. Acad. Sci. 95*:4941-4946 (1998); Holtmann, M., *et al.*, *J. Biol. Chem. 270*:14394-14398 (1995); DeAlmeida, V. and Mayo, K., *Mol. Endo. 12*:750-765 (1998)) joined to Glu-182 (Figure 3B). COS-7 cells were transfected in 24-well plates when the cells were 85 to 95% of confluency, using 200 ng of plasmid DNA that was purified by cesium chloride/ethidium bromide gradient centrifugation for each well. Assays were conducted 72 to 96 hours after transfection. Under these conditions about ~20 % of the COS-7 cells become transfected and express about $5x10^6$ surface PTH receptors per cell (Bergwitz, C., *et al.*, *J. Biol. Chem. 272*:28861-28868)).

[0077] Intracellular cyclic AMP: Transfected COS-7 or HKRK-B7 cells were rinsed with 500 ml of binding buffer, (50 mM Tris-HCl, pH 7.7, 100 mM NaCl, 5 mM KCl, 2 mM CaC12, 5% heat-inactivated horse serum, 0.5% heat-inactivated fetal bovine serum) and 200 ml of IBMX buffer (DMEM containing 2 mM IBMX, 1 mg/ml bovine serum albumin, 35 mM Hepes-NaOH, pH 7.4) and 100 ml of binding buffer or binding buffer containing various amounts of peptide were added. The plates were incubated for 60 minutes at room temperature. The buffer was then withdrawn and the cells were frozen on dry ice, treated with 0.5 ml of 50 mM HCl, and refrozen. After

the thawing, the lysate was diluted 30-fold in dH20 and an aliquot was analyzed for cAMP content by determined radioimmunoassay using unlabeled cAMP as a standard.

[0078] For cAMP inhibition assays, transfected COS-7 cells were rinsed once with 500 ml of binding buffer, and 200 ml of IBMX buffer and 100 ml of binding buffer or binding buffer containing the antagonist [Leu11,D-Trp12]hPTHrP(7-34) NH2 (10 mM) were added. After a 5 minute incubation at room temperature, 10 ml of binding buffer containing PTH(1-14) or PTH(1-34) (agonist peptide) was added, and the incubation was continued for an additional 30 minutes. The cells were then lysed and intracellular cAMP levels were measured as described above.

EXAMPLE 1

PTH(1-14) action in stable cells.

[0079] Amino-terminal peptide fragments based on the rat PTH sequence and ranging in length from PTH(1-9) to PTH(1-15) were synthesized and tested for activity in an LLC-PK1-derived cell line called HKRK-B7 which stably expresses high levels ($1x10^6$ receptors/cell) of the cloned human PTH-1 receptor (Takasu, H. and Bringhurst, F., *Endocrinology*, in press (1998)). As shown in Figure 1A, the intact control peptide PTH(1-34) mediated a 50-fold increase in intracellular cAMP levels relative to the basal cAMP level, and the estimated EC50 for this response was ~2nM with PH(1-13)and shorter fragments little or no increase in cAMP accumulation was observed (Figure 1A). However, two of the amino-terminal fragments, PTH(1-14) and PTH(1-15), stimulated cAMP formation to about 20-fold over the basal level, although the doses required for this activation were five to six orders of magnitude higher than the dose required for PTH(1-34). The response to these active peptides was dependent on the transfected PTH receptor, as parental LLC-PK1 cells, which do not express PTH receptors, but do express the related calcitonin receptor, were unresponsive to PTH(1-34) or PTH(1-14) (Figure 2B).

[0080] With the intact receptor, the potency of PTH(1-14) was about five orders of magnitude weaker than that of PTH(1-34). This reduced potency is not surprising, given that the PTH(1-14) peptide lacks important receptor-binding residues located in the PTH(15-34) region in the PTH(15-34) domain (Nussbaum, S.R., *et al.*, *J. Biol. Chem. 255*:10183-10187 (1980); Gardella, T.J., *et al.*, *Endrocrinology 132(5)*:2024-2030 (1993); Caulfield, M.P., *et al.*, *Endocrinology 127*:83-87 (1990); and Abou-Samra, A.-B., *et al.*, *Endocrinology 125*:2215-2217 (1989)). Consistent with this, unlabeled PTH(1-14) bound too weakly to permit detection in our standard competition binding assays which used radioiodinated rPTH(1-34) as a tracer ligand, nor could we detect direct binding of radiolabled PTH(1-14) analog to the intact or truncated receptors used in this study (data not shown).

EXAMPLE 2

Alanine Scanning of PTH(1-14)

[0081] To identify residues in the PTH(1-14) fragment that play a role in activating the adenylyl cyclase-signaling pathway, an alanine-scanning approach was employed. Thirteen different alanine-substituted rat PTH(1-14) analogs were synthesized and tested for the ability to stimulate cAMP formation in HKRK-B7 cells (Figure 2). The activity profile obtained with the monosubstituted analogs revealed that residues in the 1-9 region formed a relatively intolerant segment of the peptide, whereas residues in the 10-14 region formed a comparatively tolerant segment. Thus, with the exception of Ser-3 and Ala-1 (which is the native amino-terminal residue of rat PTH) most alanine substitutions in the 1-9 region yielded peptides that were barely active or inactive. In contrast, each alanine substitution in the 10-14 region yielded peptides with activities comparable with that of native rat PTH(1-14). The activity of the alanine-3 substituted peptide correlates well with previous studies on PTH(1-34) analogs which showed that amino acids with small side chains are tolerated at this site (Cohen, F.E., *et al.*, *J. Biol. Chem. 266*:1997-2004 (1991)).

EXAMPLE 3

Potentcy of the rΔNt [[rδNt]] Receptor with Small Ligands

[0082] Experiments utilized COS-7 cells transfected with either the intact rat PTH-1 receptor (rWT-HA, Fig. 4A) or a truncated rat PTH-1 receptor with most of the amino-terminal extracellular domain deleted (rΔNt [[rδNt]], Fig. 4B). In COS-7 cells expressing rWT-HA, PTH(1-34) and PTH(1-14) mediated cAMP responses that were similar to the responses seen in HKRK-B7 cells: PTH(1-14) stimulated a 15-fold in cAMP formation, but with a potency that was four to five orders of magnitude weaker than that of PTH(1-34) (Figure 3C). Both peptides also stimulated cAMP formation in cells transfected with rΔNt [[rδNt]], but the potency of PTH(1-14) was only two orders of magnitude weaker than that of PTH(1-34) with this truncated receptor (Figure 3D). This change in the relative potency of the two ligands could be accounted for by a 100-fold decrease in the potency which PTH(1-34) exhibited with rΔNt [[rδNt]], as compared to its potency with rWT-HA, rather than a shift in the potency of PTH(1-14), which was equipotent with the two receptors (compare panels C and D of Figure 4).

[0083] Although there was no direct measurement of receptor expression in this study, the 100-fold reduction in potency that PTH(1-34) exhibited with rΔNt [[rδNt]], as compared to rWT-HA, is not likely to be due to a reduction in surface expression of the truncated receptor, since PTH(1-14) exhibited equivalent activity with rΔNt [[rδNt]] and rWT-HA (Figure 4, C and D). This suggests that the two receptors are expressed at approximately equal levels. The reduced activity of PTH(1-34) with rΔNt [[rδNt]] therefore most likely reflects a loss of important binding interactions that normally occur between the (Jüppner, H., *et al.*, *Science 254*:1024-1026 (1991), Guo, J., *et al.*, *Endocrinology 136*:3884-3891 (1995); Hruska, K.A., *J. Clin. Invest. 79*:230-239 (1987); Donahue, H.J., *et al.*, *J. Biol. Chem. 263*:13522-13527 (1988); Kolakowski, L.F., GCRDb: *A G-protein–coupled receptor Database Receptors and Channels 2*:1-7 (1994); Jüppner, H., *et al.*, *Endocrinology 134*:879-884 (1994); Lee, C., *et al.*, *Mol. Endo. 9*:1269-1278 (1995); Turner, P., *et al.*, Single Mutations Allow the PTH-2 Receptor to Respond to PTHrP J.

Bone Min. Res. 12, Supplement 1, Abstract #121 (1997); Dautzenberg, F., *et al., Proc. Natl. Acad. Sci. 95*:4941-4946 (1998), Holtmann, M., *et al., J. Biol. Chem. 270*:14394-14398 (1995); DeAlmeida, V., *et al., Mol. Endo. 12*:750-765 (1998); Stroop, S., *et al., Biochem. 34*:1050-1057 (1994); Zhou, A., *et al., Proc. Natl. Acad. Sci. USA 94*:3644-3649 (1997); Bisello, A., *et al., J. Biol. Chem. 273*:22498-22505 (1998); Bergwitz, C., *et al., J. Biol. Chem. 271*:26469-26472 (1996); Gardella, T.J., *et al., Endocrinology 135*:1186-1194 (1994); Mannstadt, M., *et al., J. Biol. Chem. 273*:16890-16896 (1998); Takasu, H. and Bringhurst, F., *Endocrinology*, in press (1998); Bergwitz, C., *et al., J. Biol. Chem. 272*:28861-28868 (1997); Lee, C., *et al., Endocrinology 135(4)*:1488-1495 (1994)) domain of the ligand and the amino-terminal domain of the receptor (Jüppner, H., *et al., Endocrinology 134*:879-884 (1994); Bergwitz, C., *et al., J. Biol. Chem. 271*:16469-26472 (1996); Mannstadt, M., *et al., J. Biol. Chem. 273*:16890-16896 (1998)). The lack of these same binding interactions could also explain the inability of PTHrP(7-34) to function as an antagonist with r$\underline{\Delta Nt}$ [[rδNt]] (see Figure 7B).

EXAMPLE 4

The r$\underline{\Delta Nt}$ [[rδNt]] Receptor Interacts with the same PTH(1-14) Functional Residues as Intact PTH-1 receptor

[0084] Experiments were designed to test whether the PTH(1-14) residues that are required for function with the truncated receptor differ from those required for function with the intact receptor. Using the alanine-scanning set of PTH(1-14) analogs, experiments tested cAMP-stimulating activity for the two rat PTH-1 receptors in COS-7 cells. As shown in Figures 5A and 5B, the activity profiles obtained with r$\underline{\Delta Nt}$ [[rδNt]] mirrored that obtained with rWT-HA, since Ser-3 and the 10-14 region of the peptide were tolerant of mutation, whereas residues 2 and 4-9 were intolerant (Figures 4A and B). Therefore, the same set of functional residues in PTH(1-14) that are required for interaction with the intact PTH-1 receptor are also required for interaction with the core domain of the receptor.

EXAMPLE 5

Specificity of Truncated Ligands and PTH-receptors

[0085] In order to test whether PTH(1-14) and rΔNt [[rδNt]] retained the appropriate recognition specificity for the corresponding parent ligand, cross-reactivity experiments were done using secretin ligands and the cloned rat secretin receptor. COS-7 cells transfected with the secretin receptor exhibited a 50-fold increase in cAMP levels in response to secretin(1-27) (1 mM), but did not respond to either PTH(1-34) (1 mM) or PTH(1-14) (100 mM) (Figure 5C). Cells expressing rΔNt [[rδNt]] responded to PTH(1-34) and PTH(1-14) but not to secretin(1-27) (1 mM) or secretin(1-13) (1 mM) (Figure 5B). Thus, the recognition specificity of PTH(1-14) and rΔNt [[rδNt]] appear to replicate that of the intact parent molecules. No evidence for relaxed specificity was detected in these studies (Figure 6). It is also worth noting that PTH(1-14) did not activate the endogenous calcitonin receptors expressed in LLC-PK1 cells (Figure 2B).

EXAMPLE 6

rΔNt [[rδNt]] Stimulation is Not Affected by the Inhibitor [Leu11,D-Trp12]hPTHrP(7-34)NH2

[0086] In order to determine whether [Leu11,D-Trp12]hPTHrP(7-34)NH2, a potent competitive antagonist of PTH(1-34) action (Nutt, R.F., *et al.*, *Endocrinology 127*:491-493 (1990)), could block the ability of PTH(1-14) to stimulate cAMP formation in COS-7 cells expressing either rWT-HA or rΔNt [[rδNt]] (Figure 6). With rWT-HA the inhibitor peptide reduced the efficacy of both PTH(1-14) and PTH(1-34) by as much as 70 % as compared to the responses elicited by these agonists in the absence of inhibitor (Figure 6A). In contrast, PTHrP(7-34) had little or no effect on the ability of PTH(1-34) or PTH(1-14) to stimulate cAMP production in cells expressing rΔNt [[rδNt]].

[0087] The ability of PTHrP(7-34) to antagonize PTH(1-14) action on the intact receptor (Figure 6A) suggests that the receptor sites occupied by these two ligands overlap. This overlap could involve ligand residues 7-14 and some portion in the core region of the receptor. Any binding interactions that

may occur between figure residues (7-34) and the core region of the receptor are, however, too weak to enable effective antagonism in the absence of the amino-terminal extracellular receptor domain.

[0088] These findings establish that a much smaller region of PTH(1-34) than heretofore appreciated can stimulate receptor activation, and that the amino-terminal portion of PTH the hormone interacts with the core region of the receptor containing the seven transmembrane helices and connecting loops, as previously hypothesized for intact PTH ligands and receptors (Lee, C., *et al.*, *Mol. Endo. 9*:1269-1278 (1995); Bisello, A., *et al.*, *J. Biol. Chem. 273*:22498-22505 (1998); Bergwitz, C., *et al.*, *J. Biol. Chem. 271*:26469-26472 (1996); Gardella, T.J., *et al.*, *Endocrinology 135*:1186-1194 (1994); Bergwitz, C., *et al.*, *J. Biol. Chem. 272*:28861-28868; Gardella, T., *et al.*, *J. Biol. Chem. 271*:12820-12825 (1996)). Furthermore, this component of the interaction is sufficient for receptor signaling. The hypothesis that the 15-34 region of PTH binds to the amino-terminal extracellular domain of the receptor does not exclude the possibility that this domain, which by itself does not stimulate cAMP formation (data not shown), also provides some binding energy by interacting with the core region of the receptor. In fact, the ~100-fold greater potency that PTH(1-34) exhibits with <u>rΔNt</u> [[rδNt]], in comparison to the potency of PTH(1-14) with this receptor (Figure 4D), might well be due to such interactions. However, we can not exclude the alternative possibility that the 15-34 domain enhances the intrinsic signaling activity of the (1-14) segment, for example, by stabilizing a favorable secondary structure in the amino-terminal portion of the ligand. More specific information on the receptor recognition sites utilized by PTH and the structure of the receptor-bound ligand are required to distinguish between such possibilities.

[0089] Some recognition determinants have been identified in the amino-terminal extracellular domain, the extracellular loops and the transmembrane helices of the B family of receptors (Turner, P., *et al.*, Single Mutations Allow the PTH-2 Receptor to Respond to PTHrP J. Bone Min. Res. 12, Supplement 1, Abstract #121 (1997), Dautzenberg, F., *et al.*, *Proc. Natl. Acad. Sci. 95*:4941-4946 (1998), Holtmann, M., *et al.*, *J. Biol. Chem. 270*:14394-14398 (1995), Gardella, T.J., *et al.*, *Endrocrinology 135*:1186-1194 (1994);

Bergwitz, C., *et al.*, *J. Biol. Chem. 272*:28861-28868; Turner, P.R., *et al.*, *J. Biol. Chem. 271(16)*:9205-9208 (1996)).

[0090] One distinguishing feature of the family B receptors is the amino-terminal extracellular domain, which is relatively large and contains a number of conserved residues, including six cysteines. It is thus intriguing that this domain of the PTH-1 receptor is not essential for ligand-dependent signal transduction, as evidenced by the results with the rΔNt [[rδNt]] receptor.

[0091] Several other reports on other family B receptors provide additional evidence to suggest that the amino-terminal extracellular domains of these receptors may not be essential for functional expression. Large amino-terminal deletions in the calcitonin receptor (Unson, C., *et al.*, *J. Biol. Chem. 270*:27720-27727 (1995)) and in the growth hormone-releasing factor receptor (DeAlmedia, V. and Mayo, K., *Mol. Endo. 12*:750-765 (1998)) were compatible with expression, as assessed by immunologic methods, and a glucagon receptor lacking the amino-terminal domain and containing an activating mutation in helix 2 (HR-178) exhibited constitutive cAMP-signaling activity (Hjorth, S., *et al.*, *Mol. Endo. 12*:78-86 (1998)). In these studies, however, evidence that the truncated receptor could interact with ligand. as we have found for the PTH-1 receptor, was not reported. In a separate study on the lutropin receptor, a group A receptor that binds the large glycohormone human choriogonadotropin, it was observed that a deletion of the large amino-terminal extracellular domain yielded a receptor that could mediate a cAMP response to high doses of hCG (Ji, I.H. and Ji, T.H., *J. Biol. Chem. 266(20)*:13076-13079 (1991)).

[0092] That the activily of PTH(1-14) was not affected by the deletion of the amino-terminal receptor domain suggests that the peptide interacts predominantly with the core region of the receptor. This conclusion is supported by the alanine-scanning experiments performed on PTH(1-14), in which the profile of tolerant and intolerant residues observed with rΔNt [[rδNt]] was nearly the same as that obtained with the intact receptor (Figure 4). With each receptor, residues in the 10-14 region of the ligand formed a tolerant segment, while residues in the 1-9 region, excluding 1 and 3, formed an intolerant segment. This pattern of critical and non-critical residues observed in the context of the PTH(1-14) fragment closely matches the

patterns found previously in studies on longer-length PTH analogs (Cohen, F.E., *et al.*, *J. Biol. Chem. 266*:1997-2004 (1991); Gombert, F., *et al.*, in "Peptides: Chemistry, Structure and Biology Proceedings of the 14th American Peptide Symposium June 18-23, Kaumaya, P. and Hodges, R., eds., pp. 661-662, *Mayflower Scientific Limited*, Kingswinford, UK (1996); Gardella, T.J., *et al.*, *J. Biol. Chem. 266*:13141-13146 (1991)).

[0093] It is to be appreciated that the Detailed Description section, and not the Summary and Abstract sections, is intended to be used to interpret the claims. The Summary and Abstract sections may set forth one or more but not all exemplary embodiments of the present invention as contemplated by the inventor(s), and thus, are not intended to limit the present invention and the appended claims in any way.

PTH RECEPTOR AND SCREENING ASSAY UTILIZING THE SAME

ABSTRACT

The invention provides a novel PTH receptor polypeptide, rΔNt [[rδNt]], characterized by a deletion of the extracellular amino-terminus, ligand binding domain of the receptor. Additionally disclosed are nucleic acid molecules encoding the receptor. The receptor has a minimal domain for ligand binding and is useful in screening assays designed for the identification of agonists and antagonists of PTH receptor activity.

EXHIBIT 1

		Due Date:	None
Applicants:	Gardella *et al.*	**Art Unit:**	1645
		Examiner:	*To Be Assigned*
Application No.:	09/869,565	**Docket:**	0609.4730000
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For:	**PTH Receptor and Screening Assay Utilizing the Same**		

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EXHIBIT 2

Bioactivities and Secondary Structures of Constrained Analogues of Human Parathyroid Hormone: Cyclic Lactams of the Receptor Binding Region†

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In a search for analogues of human parathyroid hormone (hPTH) with improved activities and bioavailabilities, we have prepared the following three lactam analogues of hPTH-(1−31)-NH_2 (**1**) or [Leu^{27}]hPTH-(1−31)-NH_2 (**2**): [Leu^{27}]*cyclo*(Glu^{22}−Lys^{26})-hPTH-(1−31)-NH_2 (**3**), [Leu^{27}]-*cyclo*(Lys^{26}−Asp^{30})-hPTH-(1−31)-NH_2 (**4**), and *cyclo*(Lys^{27}−Asp^{30})-hPTH-(1−31)-NH_2 (**5**). Analogues **1**, **2**, and **5** had seven or eight residues of α-helix, as estimated from their circular dichroism (CD) spectra, in contrast to 12 residues in cyclic analogues **3** and **4**. Thus, lactams **3** and **4** stabilized a helix previously shown to exist within residues 17−29. The adenylyl cyclase activity (EC_{50}), measured in rat osteosarcoma 17/2 cells, of **5** (40.3 ± 2.3 nM) was half that of its linear form **1** (19.9 ± 3.9 nM). The linear Leu^{27} mutant **2** was twice as active (11.5 ± 5.2) as analogue **1**, and lactam analogue **3** was 6-fold more active (3.3 ± 0.3 nM). Lactam analogue **4** had less activity (16.9 ± 3.3 nM) than **2**, its linear form. Peptides hPTH-(1−30)-NH_2 (**6**), [Leu^{27}]hPTH-(1−30)-NH_2 (**7**), and [Leu^{27}]*cyclo*(Glu^{22}−Lys^{26})-hPTH-(1−30)-NH_2 (**8**) all had AC-stimulating activities similar to that of **1**. When injected intravenously, with a dose of 0.8 nmol/100 g of analogue in acid saline, hypotensive effects paralleled their adenylyl cyclase activities. They behaved quite differently when applied subcutaneously. Analogues **1**, **5**, and **6**, the weakest, showed about half the drop in blood pressure observed with **3** and **4**, the most active. In contrast, the time required to reach a maximum drop in blood pressure of **4**−**8**, after subcutaneous administration, was 2−4 times that of the other analogues. Thus, the bioavailabilities of the lactam analogues, unlike their adenylyl cyclase-stimulating activities, were highly dependent on the presence or conformation of Val^{31}.

Introduction

Parathyroid hormone (PTH) is a major regulator of extracellular calcium through its action on osteoblast and renal epithelial cell receptors[1] and can have both catabolic and anabolic effects on bone. There has recently been renewed interest in PTH as an anabolic agent for the treatment of osteoporosis. Thus more potent analogues of PTH and analogues with increased bioavailabilities may be very attractive for therapeutic use.

The biological activities of PTH are almost entirely restricted to a 34-residue N-terminal fragment of the 84-residue holohormone.[2] On binding to its receptor, this peptide stimulates both adenylyl cyclase (AC)[1] and protein kinase C's (PKC)[3−5] activities. The latter effect results from a stimulation of phosphatidylinositol-specific phospholipase-Cβ (PLC) with a subsequent stimulation of PKC by diacylglycerols released by PLC-induced phospholipid breakdown. Although even shorter C-terminal truncated fragments have some residual activity,[6,7] the minimal sequence for full AC activity is PTH-(1−28)-NH_2.[7] In contrast, stimulation of PKC's activity has been shown to require only the residue's 29-32 amide.[5] Consequently, the AC-stimulating and PKCs-stimulating activities of PTH can be separated. Thus, hPTH-(1−31)-NH_2 has no PLC/PKC-stimulating activity, but stimulates AC in osteoblasts[5] and bone growth in ovariectomized rats.[8,9]

Early CD studies indicated the presence of some ordered structure within PTH-(1−34).[10] Further CD studies of fragments of PTH-(1−34) and the holohormone, hPTH-(1−84), in neutral, aqueous buffer, showed this α-helical structure to be within residues 16−31.[11,12] NMR studies of fragments of hPTH-(1−84) have shown a general absence of structure in neutral, aqueous buffer, but residues 17−29 formed an α-helix on the addition of about 10% trifluoroethanol (TFE).[14] Higher concentrations of TFE stabilize additional α-helix near the N-terminus of 1−34.[13,15−17] CD studies of PTH fragments in the presence of lipid vesicles further showed the presence of an amphiphilic helix within residues 20−34.[11] This amphiphilic helix partially overlaps the helix that is stable in the absence of lipid. Very recently, an NMR study of hPTH-(1−37) has shown this analogue to have a secondary and tertiary structure in a near neutral solvent.[18]

Receptor-binding studies of PTH fragments have indicated a principal binding region within residues 14−34.[19] We have suggested that the 17−29 α-helix of the residue binds as such to the PTH receptor and that the amphiphilic portion of this α-helix binds with its hydrophobic face to the receptor.[7] This model is consistent with the results of a study of receptor binding region analogues.[12]

† Abbreviations: PTH, parathyroid hormone; hPTH, human parathyroid hormone; CD, circular dichroism; NMR, nuclear magnetic resonance; Fmoc, 9-fluorenylmethoxycarbonyl; TBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; HOBt, 1-hydroxybenzotriazole; TFA, trifluoroacetic acid; NMM, *N*-methylmorpholine; DCM, dichloromethane; AC, adenylyl cyclase; PKC, protein kinase Cs; PIPLC, phosphatidylinositol-specific phospholipase-Cβ; EC_{50}, effective concentration for half-maximal activity; SEM, standard error of the mean; TFE, trifluoroethanol.

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NMR studies have shown that even a model peptide found to be highly helical by CD also populates many nonhelical conformations.[20] Thus, the structure of a receptor-bound peptide hormone, such as PTH, cannot be inferred reliably from its free structure in solution. Constrained analogues of peptide hormones have been used to limit the number of conformational states available to the peptide.[21] Examination of the sequence of hPTH reveals three possible salt bridges within residues 17−29 which could either stabilize or destabilize the α-helix. These are between Glu^{22} and Lys^{26}, and Lys^{26} and Asp^{30}, both of which are expected to stabilize an α-helix, and between Lys^{27} and Asp^{30}, which is expected to destabilize an α-helix.[22] Lactam formation between these residue pairs would restrict the conformations available to hPTH in this helical region. Furthermore, two of these lactams, Glu^{22}−Lys^{26} and Lys^{26}−Asp^{30}, which are expected to stabilize the α-helical structure, are located on the polar face of the amphiphilic portion of the α-helix. The third one, Lys^{27}−Asp^{30}, is expected to at least partially destabilize the α-helix and involves a residue, Lys^{27}, which is on the hydrophobic face of the amphiphilic helix.

In this paper, we present data on the effects of these lactams on structure and bioactivities. We have used CD to monitor the α-helical secondary structure and, for bioactivities, measured AC stimulation and hypotensive activities in rats. These latter measurements give an indication of the relative stabilities and bioavailabilities *in vivo* of these analogues. The data are consistent with hPTH binding to its receptor as an α-helix with its hydrophobic face toward the receptor. Furthermore, the presence of the lactams can profoundly affect their *in vivo* activities and availabilities.

Results

Circular Dichroism Spectroscopy. Three of the lactams used in this study are derivatives of either hPTH-(1−31)-NH_2 (**1**) or [Leu^{27}]hPTH-(1−31)-NH_2 (**2**) (Figure 1). The CD spectra of [Leu^{27}]*cyclo*(Glu^{22}−Lys^{26})-hPTH-(1−31)-NH_2 (**3**) and [Leu^{27}]*cyclo*(Lys^{26}−Asp^{30})-hPTH-(1−31)-NH_2 (**4**) are shown in Figure 2, together with the spectrum of the parent linear analogue, **2**. The CD spectra of both of the lactams in Figure 2 are substantially different than that of their linear analogue. In contrast, the spectra of the Lys^{27}−Asp^{30} lactam **5** is quite similar to the spectrum of its linear species, **1** (Figure 3).

Interpretation of the CD of small peptides is not straightforward. In part, this is because small peptides usually exist in a multitude of similar conformers.[23] The CD spectrum of a polypeptide is particularly sensitive to the presence of an α-helix, and the inference of the α-helix from the spectrum is most reliable. However, the length dependency of CD signals of peptides and the possible contributions of aromatic residues to the CD signal results in an uncertainty in quantitative interpretations.[24] Since estimating the secondary structure of short peptides from their CD spectra with algorithms which use basis sets derived from protein structures is not valid, we have previously used the ellipticity at 222 nm, $[\theta]_{222}$, to measure the α-helix content in PTH analogues.[7,11,25] This is because the rotational strength of the n−π* transition at 222 nm is particularly strong in the α-helix[26] as compared to other peptide secondary structures.

1 5 10 15 20 25 30

(1) S V S E I Q L M H N L G K H L N S M E R V E W L R K K L Q D V-NH_2

(2) S V S E I Q L M H N L G K H L N S M E R V E W L R K L L Q D V-NH_2

(3) S V S E I Q L M H N L G K H L N S M E R V E W L R K L L Q D V-NH_2

(4) S V S E I Q L M H N L G K H L N S M E R V E W L R K L L Q D V-NH_2

(5) S V S E I Q L M H N L G K H L N S M E R V E W L R K K L Q D V-NH_2

(6) S V S E I Q L M H N L G K H L N S M E R V E W L R K K L Q D-NH_2

(7) S V S E I Q L M H N L G K H L N S M E R V E W L R K L L Q D-NH_2

(8) S V S E I Q L M H N L G K H L N S M E R V E W L R K L L Q D-NH_2

Figure 1. Sequences of hPTH analogues: **1**, hPTH-(1−31)-NH_2; **2**, [Leu^{27}]hPTH-(1−31)-NH_2; **3**, [Leu^{27}]*cyclo*(Glu^{22}−Asp^{30})-hPTH-(1−31)-NH_2; **4**, *cyclo*(Lys^{26}−Asp^{30})[Leu^{27}]hPTH-(1−31)-NH_2; **5**, *cyclo*(Lys^{27}−Asp^{30})-hPTH-(1−31)-NH_2; **6**, hPTH-(1−30)-NH_2; **7**, [Leu^{27}]hPTH-(1−30)-NH_2; **8**, *cyclo*(Glu^{22}−Lys^{26})[Leu^{27}]hPTH-(1−30)-NH_2.

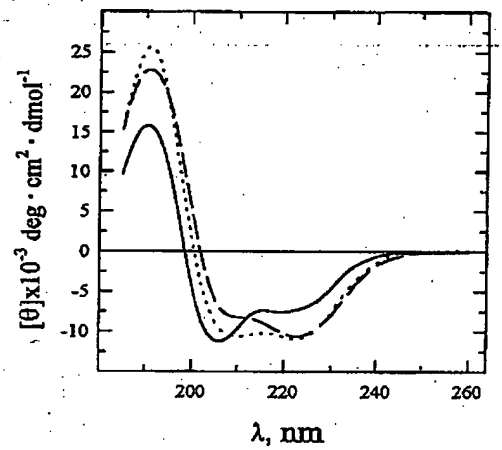

Figure 2. CD spectra of *i*, *i* + 4 lactams. Spectra were taken in 25 mM sodium phosphate, pH 7.2, at 20 °C. Shown are peptides **2** (—), **4** (− − −), and **3** (- - -). Concentrations were 0.35 mg/mL (**2**), 0.22 mg/mL (**3**), and 0.24 mg/mL (**4**).

The two lactams with *i*, *i* + 4 spacings, peptides **3** and **4**, are expected to be helix stabilizing.[21] They have similar values of $[\theta]_{222}$, approximately −10 600 and −12 600 (deg/cm²)/dmol⁻¹, respectively, and the corresponding estimates of α-helical residues are 11 and 13, compared with eight residues of the α-helix in the linear form, peptide **2**. Although they have similar intensities at 222 nm, the spectra of these two lactams are in other respects quite different. In contrast, the similarity of the CD spectra of **5** to its linear form, **1**, suggests that their average conformations are quite similar. The cyclic form is estimated to have seven residues as the α-helix, slightly less than the estimate of eight for the linear form.

To compare these spectra with that of a commonly used α-helix spectrum,[26] we have used the ratios of the absolute values of $[\theta]_{192}$ to $[\theta]_{222}$, and $[\theta]_{222}$ to $[\theta]_{209}$. These ellipticities derive from characteristic minima at 209 and 222 nm, and a maximum at 192 nm for a pure

Table 1. Helical Parameters of hPTH Analogues and a Standard α-Helical Spectrum

analogue[a]	$[\theta]_{222} \times 10^{-3}$	$\lvert[\theta]_{192}/[\theta]_{222}\rvert$	$\lvert[\theta]_{222}/[\theta]_{209}\rvert$
standard α-helix		2.63	1.09
hPTH-(1−31)-NH_2	−7.55	1.26	0.67
[Leu[27]]hPTH-(1−31)-NH_2	−7.35	2.07	0.72
[Leu[27]]*cyclo*(Lys[26]−Asp[30])-hPTH-(1−31)-NH_2	−10.6	2.14	1.30
cyclo(Lys[27]−Asp[30])-hPTH-(1−31)-NH_2	−6.34	1.19	0.69
[Leu[27]]*cyclo*(Glu[22]−Lys[26])-hPTH-(1−31)-NH_2	−12.6	2.32	1.03

[a] hPTH analogues were measured in 25 mM sodium phosphate, pH 7.2. [b] Ratios taken from α-helix data of Yang et al.[27]

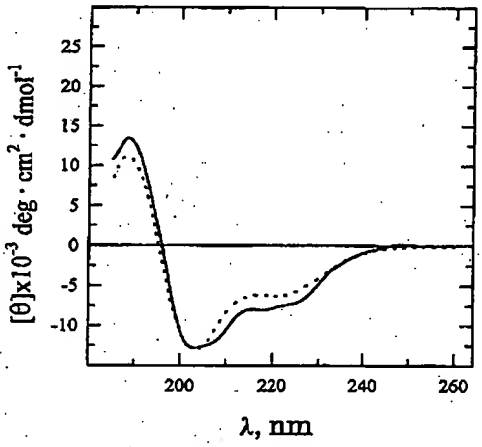

Figure 3. CD spectrum of $i, i+3$ lactam. Spectra were taken in 25 mM sodium phosphate, pH 7.2, at 20 °C. Shown are peptides **1** (—) and **5** (- - -). Concentrations were 0.13 mg/mL (**1**) and 0.35 mg/mL (**5**).

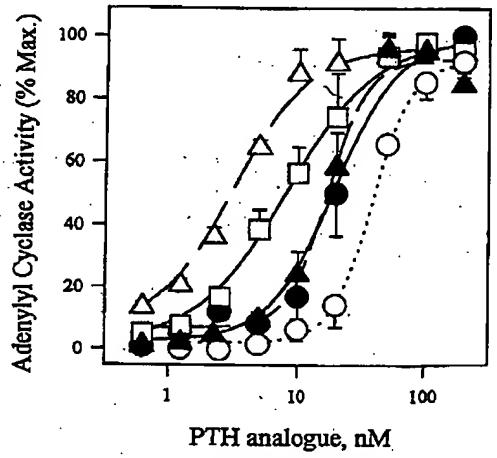

Figure 4. Stimulation of adenylyl cyclase by cyclic hPTH analogues. Shown are peptides **1** (●); **2** (□); **3** (△); **4** (▲); **5** (○).

α-helix. The ratios for the standard spectrum are 2.63 and 1.09 for $[\theta]_{192}/[\theta]_{222}$ and $[\theta]_{222}/[\theta]_{209}$, respectively.[26] In the spectrum of each of **2**, **3**, and **4**, the maximum occurs at about 190 nm, and one minimum is at 222−224 nm. However, the other minimum, which occurs at 203.5 nm in the linear peptide **2**, is shifted to 210.6 nm in the 26−30 lactam **4** and to 210.0 nm in the spectrum of the 22−26 lactam **3**. This minimum is characteristically at 209 nm in an α-helical spectrum. This CD difference between peptide **2** and peptides **3** and **4** could result from less contribution from a more "random" structure, as we have previously speculated,[11] or from the adoption of a more perfect α-helical structure in the region which tends to be α-helical, residues 17−29. Using the characteristic helical parameters mentioned above, these two lactams are definitely more helical or in a more perfect helix than the corresponding linear peptide (Table 1). Furthermore, based on the $[\theta_{222}]$ to $[\theta_{209}]$ ratio, the 22−26 analogue **3** adopts a more perfect helix than the 26−30 analogue **4**. Even though a detailed secondary structure cannot be inferred from these CD spectra, they clearly are in agreement with the expected stabilization of α-helical structure on formation of these lactams. The apparent difference in the conformation stabilized by the lactams of peptides **3** and **4** likely relates to their position in the helix.

Adenylyl Cyclase Activities. We previously reported that [Leu[27]]hPTH-(1−34)-NH_2 is more active in stimulating AC activity in the ROS cell line than hPTH-(1−34)-NH_2.[27] We have also found that peptide **2** (EC_{50}, 11.5 ± 5.2 nM) is more active than the native sequence **1** (EC_{50}, 19.9 ± 3.9 nM) (Figure 4). Lactam formation between Glu[22] and Lys[26] (**3**) induced a still greater AC-stimulating activity, with EC_{50} values of 3.3 ± 0.3 nM (Figure 4). Thus, the net effect of this cyclization and replacement of Lys[27] with Leu is about a 6-fold increase in activity. In contrast, lactam formation between either Lys[26] and Asp[30] (**4**) or Lys[27] and Asp[30] (**5**) resulted in a lessening of adenylyl cyclase stimulation, with respect to their parent linear sequences. Thus, the 26−30 lactam (**4**) has slightly less activity than its linear form, with an EC_{50} of 17.0 ± 3.3 nM vs 11.5 ± 5.2 nM for **2**. The 27−30 lactam (**5**) more markedly reduces the activity of the parent linear peptide, having an EC_{50} of 40.3 ± 2.3 nM as compared to 19 ± 3.9 nM for **1**.

We have previously reported that hPTH-(1−30)-NH_2 (**6**) has an AC-stimulating activity (EC_{50}, 20 nM) close to that of analogue **1**. We have now found that [Leu[27]]-hPTH-(1−30)-NH_2 (**7**) and *cyclo*(Glu[22]−Lys[26])-hPTH-(1−30)-NH_2 (**8**) have similar AC-stimulating activities to peptide **6**.

Hypotensive Effects of Lactam Analogues. AC activation by PTH is essential for both stimulating bone formation in ovariectomized rats[8,9,28] and reducing blood pressure.[29,30] Indeed, we have recently shown an absolute requirement for AC-stimulating, but not PKC-stimulating, activities for the osteogenic and hypotensive action of PTH.[8,31]

The data of Figure 5 show aspects of the transient hypotensive effect of hPTH analogues when administered either intravenously or subcutaneously. When administered intravenously, all of the analogues dropped the blood pressure by about 30 mmHg in about 1−2 min. However, the blood pressure response to subcutaneously injected analogues was significantly less ($p < 0.05$) than the responses to intravenously injected analogues (Figure 5). In addition, the relative responses of the blood pressure to subcutaneously injected analogues were quite different. Thus, the effectiveness of the 22−26 (**3**) and 26−30 (**4**) lactams were not significantly different ($p > 0.05$) from that of hPTH-(1−34)-NH_2 (Figure 5).

As expected, the time required to reach the minimum blood pressure was not significantly different ($p > 0.05$)

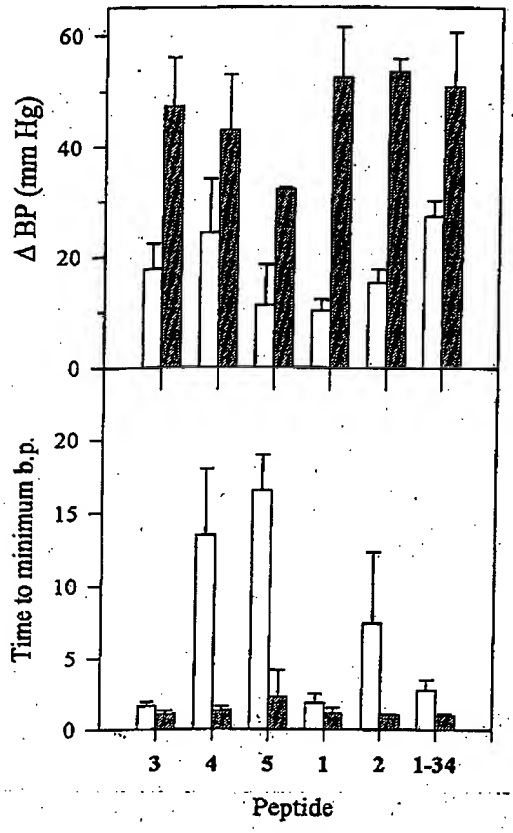

Figure 5. Hypotensive action of linear and cyclic lactam analogues of hPTH-(1−31). Shown are the maximum drops in blood pressure (upper) obtained on injecting the rat with 0.8 nmol/100 g of the analogue and the time taken to attain the maximum drop in blood pressure. The analogues (left to right, peptides **3**, **4**, **5**, **1**, **2**) were injected either intravenously (shaded bar) or subcutaneously (open bar). The graph also includes data for hPTH-(1−34)-NH_2 for comparison.

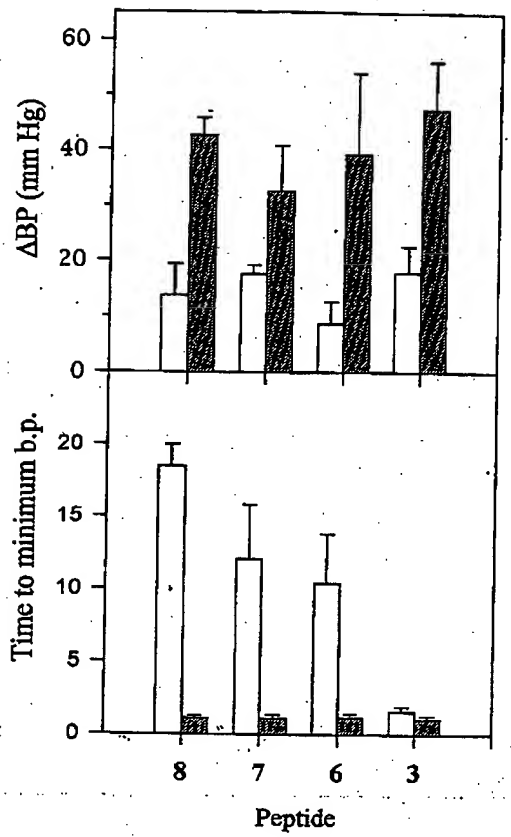

Figure 6. Hypotensive action of linear and cyclic lactam analogues of hPTH-(1−30)-NH_2 (**6**). Shown are the maximum drops in blood pressure (upper) obtained on injecting the rat with 0.8 nmol/100 g of the analogue and the time taken to attain the maximum drop in blood pressure (lower). The analogues (left to right, peptides **8**, **7**, **6**, **3**) were injected either intravenously (shaded bar) or subcutaneously (open bar).

(1−2 min) when the peptides were injected intravenously (Figure 5). In contrast, after subcutaneous injection, the times needed to reach the minimum blood pressure differed significantly ($p < 0.05$) among these analogues. Peptides **1** and **3** and hPTH-(1−34)-NH_2 rapidly decreased the blood pressure, with times of about 1.5−3 min, while peptide **2** took 7 min. In contrast, peptides **4** and **5** took much longer, about 15 min, to reach the target and affect the maximum blood pressure drop. These differences presumably reflect differing abilities of the analogues to escape from their site of injection into the blood and stimulate their vascular smooth muscle cell targets.

The low transit times observed for peptides **1** and **3**, together with the high ones for **4** and **5**, suggested that the presence of Val^{31}, and its correct conformation, might be critical to this transport of the peptide. Removal of Val^{31} resulted in all of analogues **6**, **7**, and **8** having significantly ($p < 0.05$) reduced rates of transport of the subcutaneously injected peptides to the vascular system (Figure 6). Analogues **4** and **5** have lactams involving Asp^{30}, which may restrict the conformations available to this residue. The actual total blood pressure drops of peptides **6** and **8** were, nonetheless, not significantly different ($p > 0.05$) from those of the other analogues, whether subcutaneously or intravenously administered, with the exception of peptide **5**.

Discussion

The lactam analogues described in this paper are within an amphiphilic helix[11] which is within residues 14−34, the principal binding region of PTH-(1−34).[19] Part of this region, residues 17−29, is part of an α-helix which in hPTH-(1−31)-NH_2 is partially stable in neutral, aqueous buffer.[7] This α-helix has three potential ion pairs. Although there is still debate on the contributions of such ion pairs, especially those exposed to solvent, to the stability of specific structures in peptides and proteins,[32] their presence in many of the NMR-derived models suggested that they may be making contributions to the solution structure of PTH.[18] There are two ion pairs in the C-terminal α-helical region of **1** which may contribute to its stability. These ion pairs, $Glu^{22}\cdots Lys^{26}$ and $Lys^{26}\cdots Asp^{30}$, have $i, i + 4$ spacings, and data exists suggesting pairs of these types are stabilizing.[33] The third, $Lys^{27}\cdots Asp^{30}$, with an $i, i + 3$ spacing, is expected to be destabilizing, but its effect may be minimal since it is at a terminus of the helix.[34] There is some evidence that two of these ion pairs, $Glu^{22}\cdots Lys^{26}$ and $Lys^{26}\cdots Asp^{30}$, are important to stabilizing this α-helix. The NMR structure of hPTH-(1−37) was represented by 10 model structures in the Protein Data Bank.[18] In each structure, the interionic difference of one of these potential ion pairs is 2.5 Å or less. Three models were chosen, each showing a minimal distance between one of the ion pairs, and the lactams were formed, followed by geometry optimization

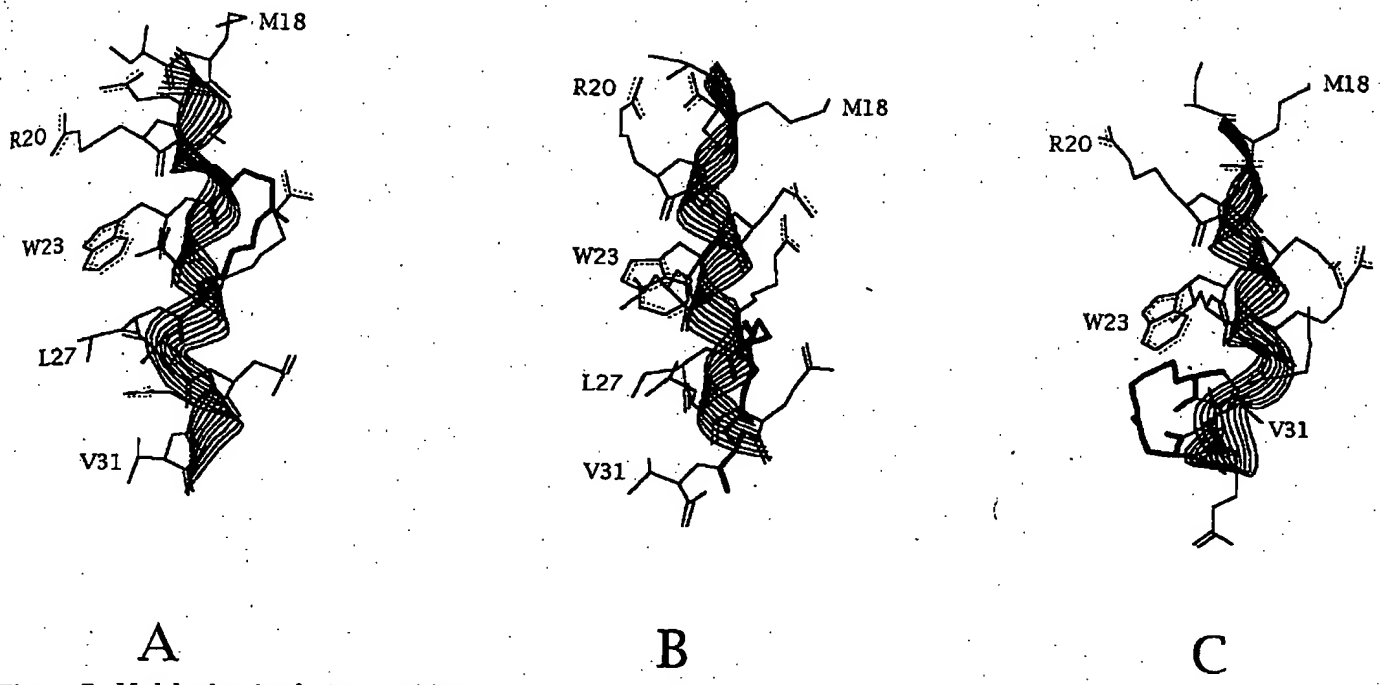

Figure 7. Models showing lactam positions in receptor-binding region of hPTH. The models were adapted from Marx et al.[18] and show the residue 17–31 region of hPTH, after lactam formation and energy minimization: A, peptide **3**; B, peptide **4**; C, peptide **5**.

by molecular mechanics to remove excess strain (Figure 7). In peptide **5** in particular, the twist introduced in the helix by the presence of the $i, i + 3$ lactam is obvious (Figure 7). This is consistent with the CD spectrum observed for **5**, and the similarity of the CD spectra of **1** and **5** suggests that the α-helix in the linear form **1** may also be somewhat frayed at the C-terminus. In the absence of the constraints introduced by the lactams, the peptide structure is dynamic, and from time to time various ion pairs exist. Each lactam has the effect of constraining the interatomic distance at the previous ion pair to 1.3 Å, thus reducing the conformational space available in the region of the lactam. The relatively small loss of AC-stimulating activity observed with analogue **5** compared to **1** implies that the receptor-binding conformation is still available after formation of the 27–30 lactam and is thus consistent with the CD observation. In contrast, the CD data of the other two lactams, **3** and **4**, indicated that each had substantially more α-helix than the common linear peptide, [Leu^{27}]-hPTH-(1–31)-NH_2. Peptide **3** had an AC-stimulating activity which was about 2.5-fold greater than the linear form. Presumably, peptide **3**, with the 22–26 lactam, stabilizes an α-helical conformer close to the receptor-binding one. In turn, the Leu^{27} analogue **2** had a greater AC-stimulating activity than the native sequence, **1**, but no increase in α-helix. However, this particular increase in AC-stimulating activity has been ascribed to a replacement of the polar Lys on the hydrophobic face of the helix by a hydrophobic Leu.[27] This result was expected if the hydrophobic face of the helix was directly involved in receptor binding and thus is not necessarily related to a stabilization of helical conformation. In contrast, peptide **4**, the 26–30 lactam of peptide **2**, had less AC-stimulating activity than its parent linear analogue, **2**.

The CD data further indicated a difference in the conformations populated by the two $i, i + 4$ spaced lactams even though they apparently had the same α-helical content. We surmised above that the 22–26 lactam analogue **3** had a more perfect helix than the 26–30 one, **4**, and this in turn may be correlated to the relative positions of each in the α-helix. Since the helical region has been shown to include residues 17–29, the Glu^{22}–Lys^{26} lactam is near the center, whereas the Lys^{26}–Asp^{30} analogue is on the periphery. It is also possible that these analogues may have differing tendencies to aggregate and that this could explain the spectral differences between peptides **3** and **4**.

The different AC-stimulating activities of peptides **3** and **4** were reflected in their hypotensive effects. When administered intravenously, each analogue elicited relative hypotensive activities not significantly different from those observed for AC-stimulating activities. This suggests that the receptors in the ROS cell line and the vascular tissue are the same. Additionally, the times to minimum blood pressure did not significantly differ. However, the results were quite different when the analogues were administered subcutaneously. Here, peptides **1**, **2**, and **5** elicited significantly lower drops in blood pressure than did hPTH-(1–34)-NH_2 and peptides **3** and **4**. Perhaps of more interest are the times observed for attaining maximal drop in blood pressure after subcutaneous administration of the various analogues. Here, the 22–26 analogue **3** reached its target much faster than either **4** or **5**, a result consistent with their data on receptor activation. The lower rates of transport of peptides **6**, **7**, and **8** further suggested that Val^{31} and the correct conformation of the peptide about Val^{31} are critical for binding to a possible transporter. The loss of charge associated with formation of the lactams at Asp^{30} is not likely to be important, since an Asp^{30} to Lys mutation was observed to have no effect on receptor-binding.[12]

The hypotensive effect measurements, especially when the analogues are administered subcutaneously, can be

affected by several variables not present in the AC-stimulating experiment. These include relative stabilities of the analogues to proteolytic degradation and transport of the analogues to the target receptor. Several workers have demonstrated increased stability to protease attack as a result of cyclization by lactam formation.[35,36] In some cases this stabilization extends beyond the lactam ring whereas in others stabilization is limited to the immediate region of the lactam ring.[36]

When administered intravenously, the relative behavior of the analogues was similar to that observed when the AC-stimulating activities were measured. Presumably, this was because a direct interaction with the vascular receptor was being measured in this case. Here, such variables as transport into the vascular system and stability were not important. However, when measurements were taken after subcutaneous injection, the relative behavior of the analogues suggested that they may be interacting with a carrier molecule during the transport process. Whatever may be the reason, it appears that formation of lactams near the C-terminus of the α-helix, such as in **4** and **5**, results in a very much increased transit time from the site of subcutaneous injection to the target receptor(s). The data demonstrate clearly that activities *in vivo* include numerous parameters not found in the *in vitro* experiment. Thus, it is valuable to have a rapid means of establishing *in vivo* capabilities, such as the hypotensive effect measurements performed in this work and previously.[31]

The PTH receptor belongs to a family of G protein-linked receptors which include such hormones as vasoactive intestinal peptide (VIP), secretin, glucagon, and calcitonin.[37] Although the peptide hormones bear no sequence similarities, the structure of the peptide as bound to the receptor may be similar. Thus, an analogue of the 28-residue VIP, *cyclo*(Lys^{21}–Asp^{25})-VIP, has more α-helix than the linear peptide and retains full bioactivity.[38] This lactam is also on the polar face of a potential amphiphilic α-helix. Interestingly, it seems that VIP and PTH may even cross-react with each other's receptors.[39] Lactam-bridged analogues of calcitonin have also been shown to have increased activities, in both *in vitro* and *in vivo* experiments. It was also stated that these increased pharmaceutical activities were compatible with an amphiphilic α-helix.[40] For this family of peptide hormones, conformational restraint from lactamization in the C-terminal region may be a general strategy for improving activities and/or bioavailabilities.

Conclusions

This work demonstrates that formation of an $i, i + 4$ lactam between residues 22 and 26 on the hydrophilic face of a C-terminal amphiphilic α-helix and substitution of a hydrophobic amino acid, Leu, for a Lys on the hydrophobic face of the helix increase the ability of hPTH-(1–31)-NH_2 to stimulate rat osteoblast adenylyl cyclase activity. This lactam stabilizes the α-helix. However, both an α-helix stabilizing lactam and a helix-destabilizing $i, i + 3$ lactam reduced the adenylyl cyclase-stimulating ability relative to the parent linear analogue. These differences in bioactivity were paralleled by the different abilities of the hPTH-(1–31)-NH_2 lactams to reduce rat blood pressure, an action that is mediated by PTH receptor-induced stimulation of vascular smooth muscle adenylyl cyclase. The data support the concept that the hormone reacts with its receptor via the hydrophobic face of an amphiphilic α-helix.

Although these peptides behave as expected for the above model, their rates of escape from subcutaneous sites of injection follow a much different pattern. Only the linear hPTH-(1–31)-NH_2, and the lactam derivative between Glu^{22}, Lys^{26} of [Leu^{27}]hPTH-(1–31)-NH_2 are able to escape rapidly from their subcutaneous sites of injection into the vascular system. Val^{31} and the conformation about Val^{31} are important for this escape. These observations may be particularly important to potential therapeutic uses of these peptides in the treatment of osteoporosis.

Experimental Section

Chemicals. *N*-α-Fmoc-L-amino acids were obtained from Novabiochem (La Jolla, CA). The following protection groups were used: Lys(Boc), Arg(Pmc), Glu(OtBu), Asp(OtBu), Ser(tBu), His(Trt), Asn(Trt), Gln(Trt), and Trp(Boc). *N*-α-Fmoc-Asp(All) and *N*-α-Fmoc-Lys(Allow) were obtained from Perceptive (Framingham, MA). Tentagel-R RAM (0.2 mmol/g) was purchased from Rapp Polymere, Tübingen, Germany. Tetrekis(triphenylphosphine)palladium(0) and sodium diethyl dithiocarbamate were from Aldrich Chemical Co. (Milwaukee, WI). Syntheses were performed on a continuous-flow peptide synthesizer (Perceptive Biosystems Model 9050 Plus). Amino acid analyses were performed with an Applied Biosystems 420H amino acid analyzer.

hPTH-(1–31)-NH_2 (1). This peptide was synthesized by a Fmoc protocol as previously described.[7] Briefly, The C-terminal Val was coupled manually to Tentagel R (0.5 g, 0.2 mmol/g). Fmoc-Val (4× molar excess) was dissolved in DMF (3.2 mL) containing 0.3 M TBTU, 0.3 M HOBt, and 0.45 M NMM, and the mixture was stirred at 45 °C for 30 min. The remaining synthesis was performed with TBTU/HOBt/NMM activation, in which the Fmoc amino acid was dissolved in 1.5 mL of DMF to a final concentration of 0.3 M and the TBTU, HOBt, and NMM concentrations were 0.26, 0.26, and 0.39 M, respectively. Flow rates were 3 mL/min. The Asn, Gln, His, Val, and Ile residues were double coupled, and a 4-fold excess of activated amino acids was used. The coupling times for Arg and Gly additions were increased from 30 to 60 min. After Fmoc removal from the N-terminal Ser, the peptide resin was washed with DCM (13 mL) and then cleaved from the resin by shaking with 7.5 mL of reagent K (6.19 mL of TFA, 0.38 mL each of water, 90% phenol/water, thioanisole, and 0.19 mL of 1,2-ethanedithiol) for 4 h at 20 °C. The cleaved peptide mixture was removed by filtration and precipitated by addition to *tert*-butyl methyl ether.

The crude product was dissolved in 14 mL of 15% acetonitrile/water and 0.1% TFA and chromatographed on a Vydac C_{18}-column (10 μm, 1 × 25 cm), using a 0.5%/min gradient (15–50%) of acetonitrile in 0.1% TFA/water. The purity of the final product was estimated by analytical HPLC on a Vydac C_{18} column (10 μm, 0.4 × 25 cm), using a 0.5%/min gradient of acetonitrile in 0.1% TFA/water. The estimated purity was >99%. The amino acid analysis is included in Table 2. The molecular weight by electrospray mass spectrometry was 3717.77(±0.13) (calculated for M + 1, 3717.14).

[Leu^{27}]hPTH-(1–31)-NH_2 (2). The peptide was synthesized and purified as above, to an estimated purity of >97%. The molecular weight was 3702.03(±1.07) (calculated for M + 1, 3703.12).

[Leu^{27}]*cyclo*(Glu^{22}–Lys^{26})-hPTH-(1–31)-NH_2 (3). This peptide was synthesized as described for hPTH-(1–31)-NH_2, with Lys-Alloc and Glu-OAll substituted at positions 26 and 22, respectively. After completion of the addition of Fmoc-Ser^{17}, the peptide-resin was removed from the column to a reaction vial (Minivial, Applied Science), suspended in 1.7 mL of a solution of tetrekis(triphenylphosphine)palladium(0) (0.24 mmol), 5% acetic acid, and 2.5% NMM in DCM under argon,

Table 2. Amino Acid Analyses of Peptides

residue	observed (expected)							
	1	2	3	4	5	6	7	8
Arg	2.3 (2)	2.2 (2)	2.1 (2)	2.2 (2)	2.3 (2)	2.1 (2)	2.1 (2)	2.2 (2)
Asp + Asn	3.4 (3)	3.4 (3)	2.9 (3)	2.9 (3)	2.9 (3)	3.0 (3)	2.6 (3)	3.1 (3)
Gly	1.1 (1)	1.1 (1)	1.2 (1)	1.1 (1)	1.2 (1)	1.0 (1)	1.1 (1)	1.0 (1)
Glu + Gln	5.2 (5)	5.3 (5)	4.7 (5)	4.7 (5)	4.6 (5)	4.6 (5)	4.3 (5)	5.0 (5)
His	2.2 (2)	2.1 (2)	2.3 (2)	2.2 (2)	2.1 (2)	2.0 (2)	2.4 (2)	2.2 (2)
Ile[a]	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)	1 (1)
Leu	5.2 (5)	6.0 (6)	5.9 (6)	6.0 (6)	5.0 (5)	5.0 (5)	6.1 (6)	5.7 (6)
Lys	3.3 (3)	2.1 (2)	2.1 (2)	1.9 (2)	2.9 (3)	3.1 (3)	2.0 (2)	2.0 (2)
Met	2.0 (2)	1.9 (2)	1.8 (2)	1.9 (2)	1.9 (2)	2.0 (2)	1.8 (2)	1.9 (2)
Ser	2.6 (3)	2.6 (3)	2.6 (3)	2.6 (3)	2.7 (3)	2.3 (3)	2.6 (3)	2.5 (3)
Trp	nd[b] (1)	nd (1)	nd (1)	nd (1)	nd (1)	nd (1)	nd (1)	nd (1)
Val	3.0 (3)	3.0 (3)	2.9 (3)	3.0 (3)	3.0 (3)	2.0 (2)	2.0 (2)	2.0 (2)

[a] Amino acid analyses ratioed to Ile. [b] Not determined.

and then shaken at 20 °C for 6 h to remove the allyl and Alloc protecting groups.[41,42] The peptide resin was then washed with 0.5% DEDT and 0.5% NMM in DMF (50 mL), followed by DMF (50 mL) and DCM (50 mL). The peptide (0.06 mmol) was cyclized by shaking with 0.06 mmol HOAt/0.12 mmol NMM in 2 mL of DMF for 14 h at 20 °C.[42,43] The peptide-resin was filtered, washed with DMF, and repacked into the column. The Fmoc-peptide was cleaved from the resin with reagent K. After the first HPLC, the Fmoc group was removed, and purification was completed by further HPLC. The purity was >99%, and the product had a mass of 3685.46(±0.46) (expected M + 1 = 3685.12). The position of the lactam was confirmed by sequencing.

[Leu^{27}]*cyclo*(Lys^{26}–Asp^{30})-hPTH-(1–31)-NH$_2$ (4). The synthesis was performed in a manner analogous to that of [Leu^{27}]*cyclo*(Glu^{22}–Lys^{26})-hPTH-(1–31)-NH$_2$. The product had an estimated purity of >95%, with a molecular weight of 3685.61(±0.36) (expected M + 1 = 3685.12). The peptide was also sequenced to confirm the lactam position.

***cyclo*(Lys^{27}–Asp^{30})-hPTH-(1–31)-NH$_2$ (5).** The synthesis was performed in a manner analogous to that of [Leu^{27}]-*cyclo*(Glu^{22}–Lys^{26})-hPTH-(1–31)-NH$_2$. The product had an estimated purity of >95%, with a molecular of 3700.64(±0.38) (expected M + 1 = 3700.14). The peptide was sequenced to confirm the lactam position.

hPTH-(1–30)-NH$_2$ (6). This peptide was synthesized in the same way as analogue 1, without manual addition of Val to the support. The product had an estimated purity of >99%, with a molecular mass of 3619.05(±0.48) (expected for M + 1 = 3619.01).

[Leu^{27}]hPTH-(1–30)-NH$_2$ (7). The synthesis was analogous to peptide 6. The product had an estimated purity of >99% and a mass of 3603.98(±0.19) (expected M + 1 = 3603.99).

[Leu^{27}]*cyclo*(Glu^{22}–Lys^{26})-hPTH-(1–30)-NH$_2$ (8). This peptide was synthesized as for peptide 3, without manual addition of Val to the support. The product had an estimated purity of >97%, with a molecular mass of 3586.14(±0.19) (expected M + 1 = 3685.99).

Molecular Modeling. Preliminary structures were selected from NMR data for hPTH-(1–37) stored in the Brookhaven Protein Data Bank.[18] From the models provided, selections were made of those having side-chain COOH···NH$_2$ distances close to that of the desired lactams. Specifically, models 1, 3, and 4 were used for analogues 3, 4, and 5, respectively. Manipulations on these data were performed with Hyperchem release 4 for Windows (Hypercube, Inc., Waterloo, ON, Canada). Residues 32–37 were removed and the molecule converted to a C-terminus amide. The models were subjected to a molecular mechanics energy minimization using an AMBER force field and a distance dependent electrostatic potential. These models are at a nearby local energy minimum to the initial structure and are useful in understanding the restraints imposed by the lactams. They cannot be inferred to describe the receptor-bound conformation of the analogue.

Circular Dichroism Spectroscopy. Spectra were obtained on a JASCO J-600 spectropolarimeter at 20 °C. At least four spectra were averaged and the data smoothed by the JASCO software. The instrument was calibrated with ammonium (+)-10-camphorsulfonate. Peptide concentrations were calculated from the absorption at 280 nm, using an extinction coefficient of 5700 M^{-1} for the single tryptophan. Data are expressed per peptide bond.

Adenylyl Cyclase Activities. Adenylyl cyclase activities of 4–5-day cultures of ROS 17/2 cells in 24-well plates were estimated from the rate of formation of [^{3}H]cAMP from the cellular ATP pool, which had been labeled with [^{3}H]adenine before exposure to hPTH or its analogues.[5] Cells were incubated for 10 min after addition of analogues, before stopping the reaction with 10% trichloroacetic acid, followed by separation and measurement of [^{3}H]cyclic AMP. Each experiment was done at least in duplicate, and the standard errors were determined. The values for the concentrations of half-maximal activities (EC_{50}) were determined by fitting the data to a sigmoidal function (Table Curve, Jandel, San Rafael, CA). Errors for each EC_{50} were estimated by fitting to the limits of the standard errors.

Blood Pressure Assay. Female Sprague–Dawley rats (weighing over 290 g) were anesthetized with intraperitoneally injected sodium pentobarbital (65 mg/kg of body weight).[31] Rectal temperature was monitored with a YSI402 thermistor (Yellow Springs Instrument Co., Inc., Yellow Springs, OH) and maintained between 36.0 and 38.5 °C throughout the experiment. Ear pinna temperature was also monitored using a YSI banjo thermistor. The tail artery was exposed and cannulated with a Jelco 25-g IV catheter (Johnson and Johnson Medical Inc., Arlington, TX) and connected to a Statham pressure transducer, the signals from which were recorded digitally with a Biopac Systems MP100 monitor (Harvard Instruments, Saint Laurent, QC, Canada). For intravenous injection of PTH or one of its fragments, a femoral vein was also exposed. After surgery, the blood pressure was allowed to stabilize for 8 min, after which PTH or one of its fragments (dissolved in acidified saline containing 0.001 N HCl) was injected into the femoral vein or under the skin of the abdomen. Data were collected for 12 min after intravenous injection or for 22 min after subcutaneous injection.

Blood pressure data were accumulated and averaged, using AcqKnowledge software (Biopac Systems, Santa Barbara, CA), to determine the mean changes in five animals. The standard errors were calculated from these data sets. Blood pressure data are expressed as means ± SEM's.[9] Statistical comparisons were made by one-way analysis of variance (ANOVA). When significant differences were observed, Scheffe's test was used for multiple comparisons; $p < 0.05$ was considered to be significant. No detectable change was observed when animals were injected with vehicle alone.

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PERSPECTIVE

ARTICLES

NOTES

EXHIBIT 3

Biochemistry **1990**, *29*, 1580–1586

Modifications of Position 12 in Parathyroid Hormone and Parathyroid Hormone Related Protein: Toward the Design of Highly Potent Antagonists

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ABSTRACT: Truncated N-terminal fragments of parathyroid hormone (PTH), [Tyr^{34}]bovine PTH(7–34)NH_2, and parathyroid hormone related protein (PTHrP), PTHrP(7–34)NH_2, inhibit [Nle^{8,18},[^{125}I]iodo-Tyr^{34}]-bPTH(1–34)NH_2 binding and PTH-stimulated adenylate cyclase in bone and kidney assays. However, the receptor interactions of these peptides are 2–3 orders of magnitude weaker than those of their agonist counterparts. To produce an antagonist with increased receptor-binding affinity but lacking agonist-like properties, structure–function studies were undertaken. Glycine at position 12 (present in all homologues of PTH and in PTHrP), which is predicted in both hormones to participate in a β-turn, was examined by substituting conformational reporters, such as D- or L-Ala, Pro, and α-aminoisobutyric acid (Aib), in both agonist and antagonist analogues. Except for N-substituted amino acids, which substantially diminished potency, substitutions were well tolerated, indicating that this site can accept a wide latitude of modifications. To augment receptor avidity, hydrophobic residues compatible with helical secondary structure were introduced. Incorporation of the nonnatural amino acids D-Trp, D-α-naphthylalanine (D-α-Nal), or D-β-Nal into either [Tyr^{34}]bPTH(7–34)NH_2 or [Nle^{8,18},Tyr^{34}]bPTH(7–34)NH_2 resulted in antagonists that were about 10-fold more active than their respective 7–34 parent compound. Similarly, [D-Trp^{12}]PTHrP(7–34)NH_2 was 6 times more potent than the unsubstituted peptide but retained partial agonistic properties, although markedly reduced, similar to PTHrP(7–34)NH_2. The antagonistic potentiating effect was configurationally specific. This study provides the basis for a rational approach toward the design of more potent antagonists of both PTH and PTHrP based on the introduction of hydrophobic residues (to increase receptor avidity) into sites within the antagonist sequence which have been established to be tolerant of structural manipulation.

Parathyroid hormone (PTH), a linear polypeptide of 84 amino acids, serves a critical role in calcium homeostasis. PTH receptors that stimulate adenylate cyclase and perhaps other intracellular second messengers are present in the hormone's major target tissues: kidney and bone. Recently, a new tumor-secreted hormone associated with the clinical syndrome of humoral hypercalcemia of malignancy, namely, parathyroid hormone related protein (PTHrP) (Strewler et al., 1983; Stewart et al., 1983; Rodan et al., 1983), has been structurally elucidated (Moseley et al., 1987; Stewart et al., 1987; Strewler et al., 1987; Suva et al., 1987). PTHrP and PTH are homologous in their N-terminal domains (Figure 1).

Evidence collected both in vitro and in vivo from several laboratories indicates that expression of PTHrP bioactivity results from interaction of the hormone with what has been conventionally regarded as PTH receptors. The N-terminal fragments (34 amino acids in length) of both PTHrP and PTH display similar biological profiles (Horiuchi et al., 1987; Kemp et al., 1987). Both peptides bind to and desensitize PTH receptors (Juppner et al., 1988; Fukayama et al., 1988; Rabbani et al., 1988). This similarity in bioactivity occurs despite considerable structural divergence outside of the N-terminal 13 amino acid domains.

Our laboratory has had a long-standing interest in the design of PTH antagonists. The potential research applications and clinical utility of such antagonists has been reviewed previously (Rosenblatt, 1986). The discovery of PTHrP provides new impetus for the design of potent and effective antagonists of both PTH and PTHrP based on the contribution of PTHrP to the pathogenesis of hypercalcemia of malignancy and of the new structural possibilities revealed by the PTHrP sequence.

Previous studies have shown that deletion of two amino acids from the amino terminus of [Nle^{8,18},Tyr^{34}]bPTH(1–34)NH_2, an analogue of PTH which retains full potency in most bioassays (Rosenblatt et al., 1976), yielded the most potent in vitro PTH antagonist yet described, namely, [Nle^{8,18},Tyr^{34}]bPTH(3–34)NH_2 (Rosenblatt et al., 1977). This analogue binds to bovine renal cortical membranes with K_b = 2.9 nM and inhibits PTH-stimulated adenylate cyclase with K_i = 32 nM (Goldman et al., 1988a). However, in vivo studies revealed that the analogue has weak partial agonist properties (Segre et al., 1979; Horiuchi et al., 1983a). Truncation of four more residues from the N-terminus yielded [Tyr^{34}]bPTH(7–34)NH_2. This analogue is a pure antagonist, devoid of agonist activity in vivo (Horiuchi et al., 1983b; Doppelt et al., 1986). However, its in vitro antagonist potency is about 50-fold less than that of the 3–34 analogue (Mahaffey et al., 1979; Rosenblatt et al., 1980).

The corresponding truncated N-terminal fragment of PTHrP, PTHrP(7–34)NH_2, was synthesized and found to bind to both renal and bone PTH receptors (McKee et al., 1987). It has been demonstrated to be a potent antagonist of PTHrP(1–34)NH_2 by use of the bone-derived ROS17/2.8 cells (K_b = 100 nM and K_i = 470 nM) and bovine renal membranes (K_b = 242 nM and K_i = 410 nM). However, similar to [Nle^{8,18},Tyr^{34}]bPTH(3–34)NH_2 and in contrast to [Tyr^{34}]-

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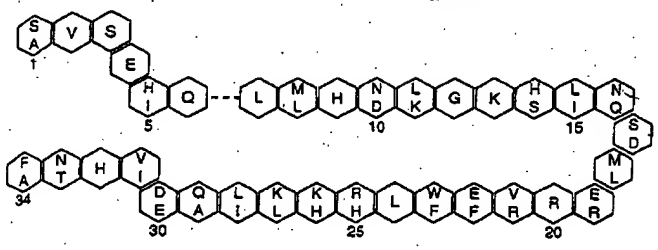

FIGURE 1: Comparison of the N-terminal sequence 1–34 of human PTH and human PTHrP. Common residues for both sequences are given by single-letter notation in the middle of the hexagon. Sites that differ in their amino acids are marked by two single letters. The letter in the upper corner of the hexagon corresponds to PTH and the letter in the lower corner to PTHrP. Deletion of six amino acids from the N-terminus eliminates a considerable portion of homology shared by the hormones.

bPTH(7–34)NH_2, PTHrP(7–34)NH_2 is a weak partial agonist (8 mM) (McKee et al., 1988).

We undertook an investigation designed to probe conformational features of PTH and PTHrP which contribute to receptor interaction. We recently compared the predicted (Chou & Fasman, 1978) secondary structure of bPTH(1–34)NH_2 and PTHrP(1–34)NH_2 utilizing a modified program (Ross & Golub, 1988). In addition to β-sheet and major α-helical regions, both peptides are predicted to contain a β-turn at positions 12–15 in PTH and 9–12 in PTHrP (see Figure 2). Experimentally, by use of circular dichroism (in water) (Brewer et al., 1974; Aloj & Edelhoch, 1972) or nuclear magnetic resonance techniques (Bundi et al., 1978), both PTH(1–84) and PTH(1–34) display mostly random structure. However, predominant helical structure is observed in nonaqueous amphiphilic environments, such as methanol (Cohn & MacGregor, 1981), and in aqueous solutions of SDS (Shah et al., 1987) or phospholipid (Epand et al., 1985).

A glycine is present in position 12 of both PTH and PTHrP. This glycine is conserved in all the known PTH sequences derived from different species. It is one of the few positions of identity for PTH and PTHrP. In addition, glycine's lack of a side chain provides conformational flexibility. Therefore, we examined the biological consequences of structural modifications at position 12. Single-residue replacements were introduced, and the biological consequences were assessed in vitro. A range of substitutions were evaluated in order to determine whether or not position 12 is important with respect to its contribution to a preferred "bioactive conformation" of the hormone or to essential interactions with receptors.

One design approach for antagonists is to identify "tolerant" positions and then introduce structural modifications that contribute additional favorable interactions with receptors that are not present in native-sequence agonists. These interactions, introduced within the framework of an antagonist sequence, might increase receptor avidity without activating receptors (transducing the transmembrane signaling apparatus).

In a recent paper (Goldman et al., 1988b), we reported the substitution of D-tryptophan for glycine at position 12 in the antagonist analogue [D-Trp^{12},Tyr^{34}]bPTH(7–34)NH_2. Inhibitory potency versus PTH-stimulated adenylate cyclase activity and binding of radioiodinated PTH agonist to PTH receptors were enhanced 12–27-fold in renal- and bone-derived systems. These findings provided impetus for more extensive and detailed examination of structure–activity relations at position 12.

In this paper, we document the identification of position 12 as a "tolerant" site and the introduction of substitutions that generate novel, highly potent PTH and PTHrP antagonists.

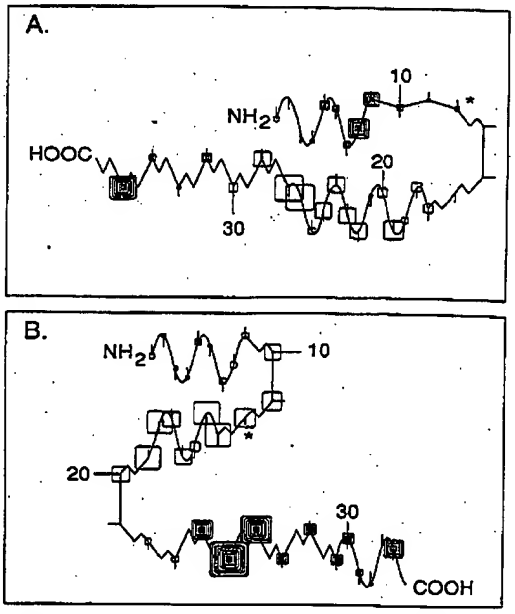

FIGURE 2: Schematic representation of secondary structure of the N-terminal 1–34 sequences of human PTH (A) and human PTHrP (B) predicted by Chou and Fasman (1978) analysis. The different structural elements are depicted as follows: sine wave, α-helix; zigzag, β-sheet; reverse direction, β-turn. Hydrophobic and hydrophilic residues are indicated by open and closed circles, respectively. The diameter of the circle is proportional to the magnitude of the hydrophobicity calculated by the method of Kyte and Doolittle (1982).

Materials and Methods

Materials. *p*-Methylbenzhydrylamine resin hydrochloride (1% cross-linked, 0.57 mM nitrogen/g, 100–200 mesh) was obtained from U.S. Biochemicals Inc. (Cleveland, OH). Ultrapure-grade [Tyr^{34}]hPTH(1–34)NH_2, [Tyr^{34}]bPTH(7–34)NH_2, [$Nle^{8,18}$,Tyr^{34}]bPTH(7–34)NH_2, *N*-Boc-L-Asp-(OcHex)-OH, *N*-Boc-N^{π}-Bom-L-His-OH, *N*-Boc-L-Nle-OH, *N*-Boc-Sar-OH, *N*-Boc-N^{in}-For-D-Trp-OH, *N*-Boc-D-Ala-OH, and *N*-Boc-β-Ala-OH were obtained from Bachem Inc. (Torrence, CA). *N*-Boc-α-aminoisobutyric acid (Aib) was obtained from Peninsula Laboratories Inc. (Belmont, CA). *N*-Boc-D-α-Nal-OH and *N*-Boc-D-β-Nal-OH were purchased from Omni Biochem (National City, CA). The rest of the *N*-Boc-protected amino acid derivatives, *N*,*N*′-dicyclohexylcarbodiimide, 1-hydroxybenzotriazole, diisopropylethylamine, trifluoroacetic acid, *N*,*N*-dimethylformamide, and dichloromethane were purchased from Applied Biosystems Inc. (Foster City, CA). Hydrogen fluoride was purchased from Matheson (Secaucus, NJ). *p*-Thiocresol, *p*-cresol, and methyl sulfide were obtained from Aldrich Chemical Co. (Milwaukee, WI). Bovine kidneys were the gift of Baums Meat Packing Inc. (Hatfield, PA). Bovine serum albumin, Tris-HCl, phosphocreatine, creatine phosphokinase, GTP, isobutymethylxanthine, and Mg-ATP were obtained from Sigma (St. Louis, MO).

PTH Receptor Binding and Adenylate Cyclase Assays. Kidney-based assays were performed with bovine renal cortical membranes following the procedures of Goldman et al. (1988a).

Bone-based assays were performed with ROS17/2.8 cells following the procedures described by McKee et al. (1988).

Constants for binding (K_b) and adenylate cyclase (stimulation, K_m; inhibition, K_i) were calculated according to Cheng and Prusoff (1973).

Peptide Synthesis, Purification, and Analytical Procedures. The analogues were synthesized by a modification of the solid-phase peptide synthetic methodology (Merrifield, 1969)

Table I: Amino Acid Analysis of the Agonists (Peptides 1–4) and Antagonists (Peptides 5–15)

amino acid residue	analogue																
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Asx	4.04[a]	4.03	4.11	4.10	4.03	4.04	4.16	2.99	3.14	3.03	2.96	3.01	3.03	2.99	3.03	2.97	1.98
	4[b]	4	4	4	4	4	4	3	3	3	3	3	3	3	3	3	2
Ser	2.99	2.95	2.98	2.94	0.96	1.08	1.15	2.04	2.03	2.28	1.91	1.99	2.09	2.13	2.10	3.03	1.04
	3	3	3	3	1	1	2	2	2	2	2	2	2	2	2	3	1
Glx	5.08	5.05	5.11	5.10	3.08	3.12	3.14	3.09	3.10	3.02	3.05	3.15	3.14	3.11	3.08	3.03	2.10
	5	5	5	5	3	3	3	3	3	3	3	3	3	3	3	3	2
Gly			1.06[c]	1.00[d]			1.01[e]										0.99
			1	1			1										1
Ala	1.03	1.01			1.00	1.05											2.01
	1	1			1	1											2
Val	2.95	2.96	2.99	2.96	1.97	1.94	2.03	1.92	1.96	2.02	1.95	1.96	1.81	1.93	1.93	1.84	
	3	3	3	3	2	2	2	2	2	2	2	2	2	2	2	2	
Ile	0.93	0.95	0.95	0.98													2.96
	1	1	1	1													3
Leu	5.15	5.11	5.09	5.13	5.00	4.89	5.25	3.97	3.93	4.13	4.02	3.90	4.15	4.09	4.07	4.29	5.03
	5	5	5	5	5	5	5	4	4	4	4	4	4	4	4	4	5
Nle													2.03	2.01	1.97	2.08	
													2	2	2	2	
Tyr	1.01	1.00	1.00	1.00	1.08	1.11	1.08	1.06	1.04	1.16	1.21	0.97	0.99	1.04	1.00	0.98	
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	
Phe								1.17	0.91	0.92	0.95	0.90	0.99	0.94	0.95	1.00	2.00
								1	1	1	1	1	1	1	1	1	2
His	2.85	3.00	3.03	3.02	2.93	2.85	2.97	3.03	2.95	3.21	3.04	3.01	2.79	2.95	2.99	2.98	3.99
	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	4
Lys	3.03	2.98	3.01	3.03	2.99	2.93	3.11	2.95	3.02	3.18	2.99	3.07	2.96	2.90	2.93	3.08	1.96
	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	2
Arg	1.94	1.94	1.73	1.73	1.95	1.94	2.06	1.95	1.95	2.06	1.94	1.98	1.94	1.92	1.94	1.99	2.92
	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	3
Met[f]	1.27	1.48	1.76	1.81	1.51	1.13	1.71	1.91	1.89	1.49	1.78	1.64					
	2	2	2	2	2	2	2	2	2	2	2	2					

[a] Values shown are the results obtained from amino acid analysis. [b] Expected values from amino acid analysis. [c] Aib substitutes Gly[12]. [d] Pro substitutes Gly[12]. [e] Met is labile under the hydrolysis conditions employed.

using an Applied Biosystems Model 430A peptide synthesizer and Version 1.2 of the software. Protection of the α-amino group was always *N*-butyloxycarbonyl (*N*-Boc). Side-chain protecting groups were as follows: N^{ϵ}-[[(2-chlorobenzyl)-oxy]carbonyl] for lysine; *O*-benzyl for glutamic acid, threonine, and serine; N^{in}-formyl for tryptophan; N^{G}-(*p*-toluenesulfonyl) for arginine; N^{π}-[(benzyloxy)methyl] for histidine. The synthesis commenced on 0.5 mmol of resin and continued, following the completion of resin-bound fully protected [Tyr[34]]bPTH(23–34) or PTHrP(20–34) sequences, on a 0.25-mmol scale. This measure was undertaken to achieve better mixing and more efficient washings of the resin. All amino acid incorporation was undertaken by a double-coupling procedure. Preformed symmetrical anhydrides were used (1 mmol per coupling cycle) except for the coupling of the protected derivatives of Asn, Gln, His, and Arg, where preformed 1-hydroxybenzotriazole esters were used (2 mmol per coupling cycle). The completed side-chain-protected resin-bound peptide was cleaved from resin with simultaneous removal of side-chain protecting groups by the low/high-HF procedure (Tam et al., 1983). The crude peptide was extracted from the residue obtained from the cleavage reaction with 50% (v/v) acetic acid, loaded on a Sephadex G-50F column (95 × 5 cm), and eluted with 50% (v/v) acetic acid at a flow rate of 1 mL/min, 10-min fractions being collected. The crude lyophilzed product obtained from the column was purified by preparative reversed-phase high-performance liquid chromatography (HPLC) using a Waters Delta Prep 3000 system and a PrePak radial compression cartridge (30 × 5.7 cm) of Vydac protein C4-bonded silica partiles of 15–20 mm and 300-Å pore size. The preparative column was eluted with a linear gradient formed by increasing the percentage of buffer B in buffer A [buffer A = 0.1% (v/v) TFA in H_2O/CH_3CN, 95:5; buffer B = 0.1% (v/v) TFA in CH_3CN] at a flow rate of 100 mL/min. The fractions (0.1 min) collected from the preparative HPLC were analyzed on an analytical reversed-phase HPLC column (15 × 0.46 cm) packed with Vydac protein C4-bonded silica particles of 5 μm and pore size of 300 Å, the same solvent system as described above being employed, but at a flow rate of 1.5 mL/min. Peptide elution was monitored at 214 nm.

A battery of analytical tests was performed to assess peptide purity for each analogue (Caporale et al., 1989). These tests included analytical reversed-phase HPLC; amino acid analysis (following 70-h hydrolysis in constant-boiling HCl) using a Beckman amino acid analyzer; fast atom bombardment mass spectrometry (FAB-MS); amino acid sequencing using an Applied Biosystems Model 470A gas-phase sequencer, to confirm the amino acid sequence and to perform "preview" analysis to assess possible contamination by deletion-containing error peptides (Tregear et al., 1977); proton nuclear magnetic resonance spectrometry using a 400-MHz instrument, to assess the presence of low-abundance side products resulting from methionine oxidation, alkylation of aromatic residues, or backbone to side-chain rearrangements.

Results

Analysis of Peptides. The amino acid analyses of all analogues synthesized and used in this study are presented in Table I. Automated Edman sequence analysis (results not shown) confirmed the presence of the desired sequence in each case, and "preview" analysis indicated the absence of deletion-containing error peptides. By use of ^{1}H NMR, no side products were detected (data not shown). The purity of the synthetic peptide analogues was >98%, determined with data from the sequencing and analytical RP-HPLC.

Biological Properties of Peptides. Shown in Table II are bovine renal receptor binding and adenylate cyclase activities

Table II: Binding and Cyclase Activity of Agonist and Antagonist Analogues Derived from PTH and PTHrP Sequences with Bovine Renal Cortical Membranes

analogue	binding, K_b (nM)	cyclase	
		K_m (nM)	K_i (nM)
(I) [Tyr^{34}]hPTH(1–34)NH$_2$	0.7 ± 0.3	0.7 ± 0.1	
(1) [Ala^{12},Tyr^{34}]hPTH(1–34)NH$_2$	1.0 ± 0.04	1.5 ± 0.2	
(2) [D-Ala^{12},Tyr^{34}]hPTH(1–34)NH$_2$	0.8 ± 0.1	1.4 ± 0.1	
(3) [Aib^{12},Tyr^{34}]hPTH(1–34)NH$_2$	0.8 ± 0.1	0.6 ± 0.2	
(4) [Pro^{12},Tyr^{34}]hPTH(1–34)NH$_2$	590 ± 200	2400 ± 770	
(II) [Tyr^{34}]hPTH(7–34)NH$_2$	260 ± 40		840 ± 180
(5) [Ala^{12},Tyr^{34}]hPTH(7–34)NH$_2$	110 ± 30		410 ± 70
(6) [D-Ala^{12},Tyr^{34}]hPTH(7–34)NH$_2$	120 ± 10		610 ± 120
(7) [Pro^{12},Tyr^{34}]hPTH(7–34)NH$_2$	470 ± 40		1400 ± 670
(III) [Tyr^{34}]bPTH(7–34)NH$_2$	80 ± 10		880 ± 70
(8) [Sar^{12},Tyr^{34}]bPTH(7–34)NH$_2$	500 ± 90		2500 ± 730
(9) [Aib^{12},Tyr^{34}]bPTH(7–34)NH$_2$	50 ± 10		550 ± 140
(10) [β-Ala^{12},Tyr^{34}]bPTH(7–34)NH$_2$	140 ± 20		300 ± 70
(11) [D-Trp^{12},Tyr^{34}]bPTH(7–34)NH$_2$	10 ± 1		70 ± 10
(12) [Trp^{12},Tyr^{34}]bPTH(7–34)NH$_2$	110 ± 10		740 ± 110
(IV) [Nle^{8,18},Tyr^{34}]bPTH(7–34)NH$_2$	150 ± 10		1550 ± 330
(13) [Nle^{8,18},D-Trp^{12},Tyr^{34}]bPTH(7–34)NH$_2$	20 ± 1		130 ± 10
(14) [Nle^{8,18},D-α-Nal^{12},Tyr^{34}]bPTH(7–34)NH$_2$	10 ± 1		350 ± 70
(15) [Nle^{8,18},D-β-Nal^{12},Tyr^{34}]bPTH(7–34)NH$_2$	10 ± 2		140 ± 40
(16) [Nle^{8,18},Trp^{12},Tyr^{34}]bPTH(7–34)NH$_2$	50 ± 3		510 ± 50
(V) PTHrP(7–34)NH$_2$	260 ± 40		610 ± 130
(17) [D-Trp^{12}]PTHrP(7–34)NH$_2$	40 ± 10		110 ± 10

of reference analogues in the agonist (I) and antagonist series (II–V) and position 12 substituted analogues of PTH (peptides **1–16**) and PTHrP (peptide **17**).

The K_b and K_m of agonist analogues containing Ala^{12}, D-Ala^{12}, and Aib^{12} (**1–3**) are in the range of 0.7–1.0 nM and 0.6–1.5 nM, respectively. These values are similar to those obtained for the parent agonist I (0.7 nM). The Pro^{12} analogue (**4**) is approximately 840- and 3500-fold less active than [Tyr^{34}]hPTH(1–34)NH$_2$ (I) in binding and adenylate cyclase assays, respectively.

In the antagonist series related to the parent analogues II–IV, analogues [Ala^{12},Tyr^{34}]- and [D-Ala^{12},Tyr^{34}]hPTH(7–34)NH$_2$, **5** and **6**, respectively, were about twice as potent as the parent peptide II in both binding and cyclase assays. Analogues **9** and **10**, modified at position 12 by Aib and β-Ala, respectively, were 2–3 times more potent in inhibiting agonist-stimulated (3 nM [Nle^{8,18},Tyr^{34}]bPTH(1–34)NH$_2$) adenylate cyclase activity than the corresponding parent antagonist III. However, analogue **10** displays lower binding affinity than the parent peptide III (cf. K_b = 140 and 80 nM, respectively). This finding is in contrast to the generally observed parallel changes in inhibition of PTH-stimulated adenylate cyclase and binding affinity for the rest of the position 12 modified analogues. The reason for this discrepancy is not known. Both imino acid containing analogues, **7** and **8**, in the antagonist series (containing Pro^{12} and Sar^{12}, respectively) display approximately one-half to one-sixth the binding affinity (470 and 500 nM for **7** and **8**, respectively, compared to 260 and 80 nM for II and III, respectively) and about 2–3-fold diminished adenylate cyclase inhibitory potency compared to the parent antagonists II and III (1400 and 2500 nM for **7** and **8**, respectively, and 840 and 880 nM for II and III, respectively). Replacement of Gly^{12} with D-Trp in III resulted in antagonist **11**, which was 10-fold more potent in the binding assay (6.7 nM) and about 12-fold more potent at inhibiting agonist-induced adenylate cyclase activity (69 nM). In contrast, introduction of L-Trp^{12} into III (antagonist **12**) failed to alter either binding affinity or inhibition of adenylate cyclase activity compared to the parent peptide. The combination of Nle^{8,18} and either D-Trp (**13**) or D-α/β-naphthylalanine (**14** and **15**, respectively) in position 12 also generated highly potent antagonists. Antagonists **13–15** were 10–14 times more potent in binding and 5–12 times more potent as inhibitors of adenylate cyclase than the corresponding parent antagonist IV. In PTHrP(7–34)NH$_2$ (V), substitution of Gly^{12} with D-Trp (analogue **17**) yielded a peptide with 6-fold greater affinity and inhibitory potency than its parent structure.

Table III: Binding and Cyclase Activity of Antagonist Analogues Derived from bPTH(7–34)NH$_2$ with Bone-Derived ROS17/2.8 Cells

analogue	binding, K_b (nM)	cyclase, K_i (nM)
(III) [Tyr^{34}]bPTH(7–34)NH$_2$	700 ± 90	2700 ± 520
(11) [D-Trp^{12},Tyr^{34}]bPTH(7–34)NH$_2$	120 ±	210 ± 120
(12) [Trp^{12},Tyr^{34}]bPTH(7–34)NH$_2$	400 ± 20	1360 ± 80
(IV) [Nle^{8,18},Tyr^{34}]bPTH(7–34)NH$_2$	960 ± 170	1550 ± 360
(13) [Nle^{8,18},D-Trp^{12},Tyr^{34}]-bPTH(7–34)NH$_2$	180 ± 30	70 ± 20
(16) [Nle^{8,18},Trp^{12},Tyr^{34}]bPTH(7–34)NH$_2$	410 ± 30	220 ± 30

Table III summarizes data for receptor binding and inhibition of PTH-stimulated adenylate cyclase activity for selected analogues in the bone-derived system. Reference analogues III and IV are compared to D- or L-Trp^{12}-substituted analogues of PTH (peptides **11–13** and **16**). Substitution of Gly^{12} with D-Trp enhances binding about 5-fold (see Table III, **11** versus III and **13** versus IV) compared to the 10-fold enhancement observed in the renal-based assay (see Table II, same analogues). The effect of this substitution on inhibition of PTH-stimulated adenylate cyclase activity in bone-derived cells is comparable to that observed in the renal membranes. Substitution by L-Trp^{12} resulted in a 2-fold increase in binding and inhibitory potency in the bone system, comparable to the effect observed for the same replacement in [Nle^{8,18},Tyr^{34}]bPTH-(7–34)NH$_2$ in the renal assays.

For agonists and antagonists, a close correlation between rank order of potency in the binding and adenylate cyclase assays is apparent. Furthermore, in the agonist series the K_b and K_i for each analogue correspond closely. For the antagonist series, however, a consistently weaker antagonist potency (about 1 order of magnitude) is observed for the K_i versus binding affinity, K_b. The reason for this discrepancy is not known.

A 3-fold difference in receptor avidity was observed for antagonists derived from human sequence versus bovine sequence (cf. II and III in Table II). Therefore, we evaluated

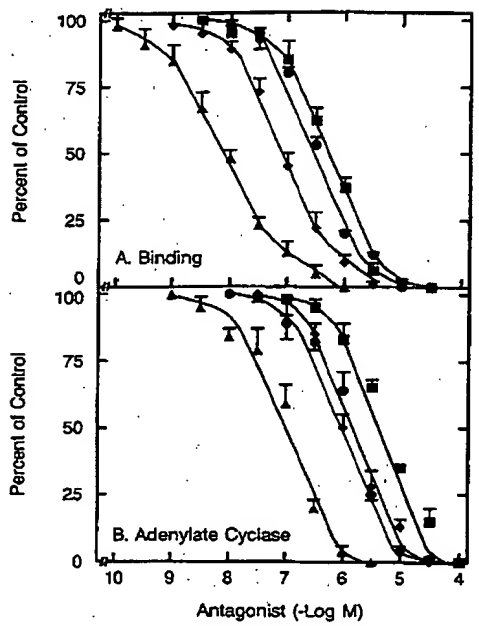

FIGURE 3: Effects of PTH and PTHrP analogues on (A) binding of radiolabeled PTH ligand, [Nle^{8,18},[^{125}I]iodo-Tyr^{34}]bPTH(1–34)NH$_2$, and (B) inhibition of PTH-stimulated adenylate cyclase in bovine renal cortical membranes. Assays were performed as described under Materials and Methods. Analogues: [D-Trp^{12},Tyr^{34}]bPTH(7–34)NH$_2$ (▲); [Tyr^{34}]bPTH(7–34)NH$_2$ (◆); PTHrP(7–34)NH$_2$ (●); [Sar^{12},Tyr^{34}]bPTH(7–34)NH$_2$ (■).

the effects of introducing modifications into both sequences. Binding and dose–response curves for PTHrP(7–34)NH$_2$ and [Tyr^{34}]bPTH(7–34)NH$_2$ and some of its analogues modified at position 12 are depicted in Figure 3.

Discussion

In order to design more potent antagonists of PTH and PTHrP, it is necessary to identify sites within the hormone molecule for which structural modification can be used to enhance affinity for the receptor without restoring agonist-like properties or diminishing specificity of receptor interaction. There are two principal ways to accomplish this objective: (1) stabilize an antagonist in a conformation favored by the receptor or (2) introduce new structural moieties that will add binding elements that interact with the receptor at sites different from those present in the native agonist. These novel binding elements should not disrupt the "bioactive conformation" required for receptor binding.

Position 12 in the PTH sequence lies outside the previously identified N-terminal "receptor-activation domain" (Rosenblatt, 1986). The studies described in this paper demonstrate that, with regard to adenylate cyclase linked receptor activity, glycine at position 12 is not essential. This position, in both a series of agonists and antagonists, tolerates a wide variety of substitutions (cf. analogues **1**, **2**, **4**–**6**, **9**, and **10**). The only substitutions demonstrated to diminish biological activity were the N-substituted amino acids, namely, Pro (analogues **3** and **7**) and Sar (analogue **8**). These findings suggest that a wide range of conformational latitude is tolerated in the region neighboring position 12, especially among residues accommodated by α-helix or β-sheet. However, known N-alkylated amino acids, which may act as helix breakers, are poorly tolerated.

This wide range of tolerance for Gly^{12} substitution is unusual and contrasts with structure–activity studies in other hormonal systems, such as cholecystokinin (Fournie-Zaluski et al., 1985), substance P (Blumberg & Teichberg, 1981), enkephalin (Walker et al., 1977), and LH-RH (Vale et al., 1977; Perrin et al., 1980), where substitutions of a wide range of amino acids at a particular site are not well tolerated.

Enhancement of hydrophobicity within the binding domain of a hormone has increased bioactivity in several hormonal systems (Manning et al., 1982a,b; Rees et al., 1974; Rivier & Vale, 1978; Fries et al., 1982; Engberg et al., 1981). Therefore, we attempted to increase antagonist activity by increasing hydrophobicity at position 12. Such modifications follow the rationale of introducing new potential receptor-binding elements into the antagonist sequence.

Analogues containing D-Trp, L-Trp, or D-α- or D-β-Nal residues in position 12 of either [Tyr^{34}]bPTH(7–34)NH$_2$ (III) or [Nle^{8,18},Tyr^{34}]bPTH(7–34)NH$_2$ (IV) were prepared (analogues **11**–**16**) and evaluated. Replacement of Gly^{12} in III by D-Trp produced the highly potent antagonist **11**, which was 12-fold more potent than III in binding and inhibition of PTH-stimulated adenylate cyclase. The similar potencies of analogues substituted by either D-Trp^{12} or D-α- and D-β-naphthylalanyl residues are in accord with observations of others regarding the functional equivalence of the indolyl and naphthyl moieties (Nestor et al., 1982, 1984). The similar potencies observed for analogues **14** and **15** containing D-α-Nal and D-β-Nal, respectively, suggest that additional hydrophobic interactions can be accommodated within a complementary domain in the receptor. This enhanced binding, however, does not extend to the increased hydrophobicity present in the original stereochemistry because the increased antagonistic properties of D-Trp^{12} substitution are lost by introducing L-Trp^{12}. This may indicate that introduction of highly hydrophobic side chains in the D configuration at position 12 provides auxillary hydrophobic interactions with the receptor that result in enhanced binding.

Along similar lines, incorporation of D-Trp in position 12 of PTHrP(7–34)NH$_2$ (V) to yield analogue **17** generated a more potent antagonist of PTHrP and reduced the partial agonism that had been observed previously in the parent antagonist (McKee et al., 1988; R. McKee, unpublished data). The disappearance of partial agonism may be attributed to the auxillary hydrophobic interaction with receptor, discussed above, which may impede assumption of a conformation required for receptor activation.

A comparison between properties observed in bovine renal cortical membranes and bone-derived rat osteosarcoma cells (ROS17/2.8) demonstrates, qualitatively, that the potency enhancement resulting from introduction of D-Trp^{12} is similar across the two systems (Tables II and III). These findings strengthen the concept that D-Trp substitution is advantageous in terms of receptor interaction because the observations are consistent across target tissues (renal vs bone), species (bovine vs rat), and nature of the preparations (membranes vs intact cells).

In conclusion, on the basis of conformational predictions, we explored the biological effects of structural modification of PTH and PTHrP at position 12. We demonstrated that position 12 in PTH tolerates a wide range of structural latitude (excluding *N*-methyl amino acids) without substantial weakening of receptor interaction. These findings permitted us to select this site for introduction of hydrphobic amino acids not present in the native PTH or PTHrP sequences. The introduction of D-Trp or other more hydrophobic (D-α/β-Nal) residues at position 12 enhances antagonist activity for analogues of the calcium-mobilizing hormones. These analogues are active in the low nanomolar range. Extension of this approach may lead to more potent PTH and PTHrP antag-

onists and, when applied to other bioactive peptides, may provide a promising direction for the rational design of potent antagonists.

ADDED IN PROOF

After submission of this paper, a publication on the structure of PTHrP(1–34) determined by NMR appeared (Barden & Kemp, 1989). The authors predict a compact structure for the 1–34 peptide with a reverse turn, formed by residues 10–13, which they also predict to be present in PTH(1–34). The authors also present data to show that PTHrP(7–34) is less structured. This decrease in structure may account for the ability of hydrophobic substitutions at position 12 to increase affinity for the receptor by permitting interaction with a novel site(s) on the receptor.

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A Model for the Interaction of Muscarinic Receptors, Agonists, and Two Distinct Effector Substances

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ABSTRACT: The binding of the agonist carbamylcholine to muscarinic receptors in rat heart myocytes from young and aged cultures and in rat atrial membranes has been measured in the absence and presence of GppNHp, pertussis toxin, and/or batrachotoxin. The effect of each of the added substances upon agonist binding was accounted for by a model according to which the receptor may form an equilibrium complex with agonist and either of two distinct effector substances, one of which is postulated to increase the affinity of receptor for agonist and the other of which is potulated to decrease the affinity of receptor for agonist.

While it is known that the binding of muscarinic agonists to their receptors inhibits the production of cyclic AMP, presumably through interaction with the adenylate cyclase system (Watanabe et al., 1978; Brown, 1979; Van Sande et al., 1977; Olianas et al., 1983), the molecular mechanism of interaction between muscarinic receptors and other membrane and intracellular components such as ion channels is as yet unclear [for reviews, see McKinney and Richelson (1984), Nathanson (1987), Sokolovsky et al. (1986), and Sokolovsky (1989)]. Because the addition of GTP and GTP analogues to membrane preparations has a significant effect on the binding of muscarinic agonists to receptors, the existence of agonist-mediated interactions between muscarinic receptors and one or more forms of G-protein is inferred (McKinney & Richelson, 1984; Nathanson, 1987; Sokolovsky et al., 1986; Sokolovsky, 1989). While classical antagonist binding isotherms are generally explicable in terms of a model for the binding of ligand to a single class of homogeneous sites (Nathanson, 1987; Sokolovsky, 1989), data characterizing the displacement of antagonist by agonist have usually required that two or three affinity classes of binding sites be postulated (Birdsall et al., 1980). Common to both the two- and three-affinity-class models has been the assumption that the fractional abundance of each class of site is independent of the concentration of agonist. The binding isotherm of agonist to n affinity classes of sites is then specified by n equilibrium association or dissociation constants and $n - 1$ fractional site abundances.

In previous studies of the effect of exogenously added substances (such as GppNHp)[1] on the affinity of receptor for agonist, the results have typically been analyzed by fitting a phenomenological two- or three-site-class model to the data (Berrie et al., 1979; Sokolovsky et al., 1980; Wei & Sulakhe, 1980; Burgisser et al., 1982; Ehlert et al., 1981; Harden et al., 1982; Walbroeck et al., 1982). If affinity for agonist is increased in the presence of the additive, the effect is "explained" as an additive-induced conversion of lower affinity sites to higher affinity sites; conversely, if affinity for agonist is decreased in the presence of the additive, the effect is "explained" as an additive-induced conversion of higher affinity sites to lower affinity sites (Birdsall et al., 1980; Sokolovsky et al., 1986). We feel that such "explanations" are merely parameterized descriptions of the data and provide little or no insight into the molecular mechanism(s) underlying the effect of exogenous additives upon agonist binding. The purpose of the present work is to present a mechanistic model that attempts to describe agonist binding and the effects of exogenous additives on agonist binding *in the context of receptor function*. Such a model must differ from a phenomenological description of agonist binding in at least two important ways:

(1) When phenomenological two- or three-site-class models are fit to a series of isotherms characterizing agonist binding to receptor in the presence of varying amounts of exogenous additive A, it is commonly observed that the affinities for agonist, as well as the abundances of the apparent site classes, vary substantially with the concentration of A (Sokolovsky et al., 1986; Sokolovsky, 1989). In contrast to a phenomenological approach to the definition of site classes, we adopt a mechanistic approach wherein total receptor is considered to exist as a population of substates. An individual substate of receptor may differ from other substates by virtue of intrinsic differences in polypeptide structure or by virtue of covalent or noncovalent association with other naturally occurring or

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[1] Abbreviations: BTX, batrachotoxin; 4NMPB, *N*-methyl-4-piperidyl benzilate; GppNHp, guanyl-5'-yl imidodiphosphate; PT, pertussis toxin.

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